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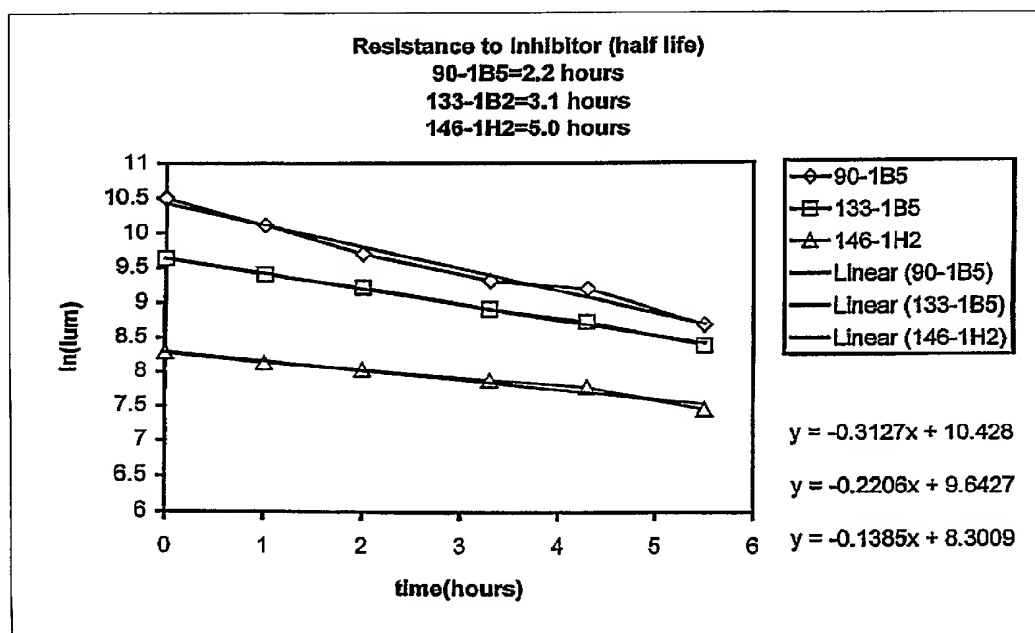
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(54) Title: THERMOSTABLE LUCIFERASES FROM PHOTURIS PENNSYLVANICA AND PYROPHORUS PLAGIOPHTHA-  
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(57) Abstract: Luciferase enzymes with greatly increased thermostability, e.g., at least half lives of 2 hours at 50 °C, cDNAs encoding the novel luciferases, and hosts transformed to express the luciferases, are disclosed. Methods of producing the luciferases include recursive mutagenesis. The luciferases are used in conventional methods, some employing kits.

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**THERMOSTABLE LUCIFERASES FROM PHOTURIS PENNSYLVANICA AND PYROPHORUS  
PLAGIOPHTHALAMUS AND METHODS OF PRODUCTION**

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**Statement of Government Rights**

The invention was made with grants from the Government of the United States of America (grants 1R43 GM506 23-01 and 2R44 GM506 23-02 from the National Institutes of Health and grants ISI-9160613 and III-9301865 from the National Science Foundation). The Government may have certain rights to the invention.

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**Field of the Invention**

The invention is directed to mutant luciferase enzymes having greatly increased thermostability compared to natural luciferases or to luciferases from which they are derived as measured, e.g., by half-lives of at least 2 hours at 50°C in aqueous solution. The invention includes mutant luciferase enzymes that are resistant to inhibition by a substrate inhibitor, e.g., a substrate analog. The invention is also drawn to polynucleotides encoding the novel luciferases, and to hosts transformed to express the luciferases. The invention is further drawn to methods of producing luciferases with increased thermostability and the use of these luciferases in any method in which previously known luciferases are conventionally employed. Some of the uses employ kits. The invention also provides a method of producing a polynucleotide sequence encoding an enzyme that is resistant to inhibition by an inhibitor, and a method which yields a polynucleotide sequence encoding an enzyme having enhanced enzymological properties.

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**Background of the Invention**

Luciferases are defined by their ability to produce luminescence. Beetle luciferases form a distinct class with unique evolutionary origins and chemical mechanisms (Wood, 1995).

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Although the enzymes known as beetle luciferases are widely recognized for their use in highly sensitive luminescent assays, their general utility has been limited due to low thermostability. Beetle luciferases having amino acid sequences encoded by cDNA sequences cloned from luminous beetles are not stable even at moderate temperatures. For example, even the most stable of the

luciferases, *LucPpe2*, obtained from a firefly has very little stability at the moderate temperature of 37°C. Firefly luciferases are a sub-group of the beetle luciferases. Historically, the term “firefly luciferase” referred to the enzyme *LucPpy* from a single species *Photinus pyralis* (*Luc +* is a mutant version of *LucPpy*, see U.S. Patent No. 5,670,356).

Attempts have been reported to mutate natural cDNA sequences encoding luciferase and to select mutants for improved thermostability (White et al., 1994; from *P. pyralis*, and Kajiyama and Nekano, 1993; from *Luciola lateralis*.) However, there is still a need to improve the characteristics and versatility of this important class of enzymes.

### **Summary of the Invention**

The invention is drawn to novel and remarkably thermostable luciferases, including luciferase enzymes with half-lives of at least 2 hours at 50°C, or at least 5 hours at 50°C, in an aqueous solution. As described hereinbelow, after 2 hours at 50°C in an aqueous solution, a thermostable luciferase of the invention lost less than 5% luminescence activity. The mutant luciferases of the present invention display remarkable and heretofore unrealized thermostability at 22°C in an aqueous solution and at temperatures at least as high as 60°C in an aqueous solution. For example, the luciferases of the invention are thermostable for at least 10 hours at 50°C; for at least 2 hours, preferably at least 5 hours, more preferably at least 10 hours, and even more preferably at least 24 hours, at 60°C; and/or for at least 100 days, preferably at least 200 days, more preferably at least 500 days, and even more preferably at least 800 days, at 22°C, in aqueous solution. For example, after 30 days at 22°C in an aqueous solution, a thermostable luciferase of the invention lost less than 5% luminescence activity. Preferably, the thermostable luciferases of the invention have enhanced luminescence intensity, enhanced signal stability, enhanced substrate utilization, and/or decreased  $K_m$ , relative to a reference, e.g., a native wild-type, luciferase. The invention is further directed to the mutant luciferase genes (e.g., cDNA or RNA) which encode the novel luciferase enzymes. The terminology used herein is, e.g., for the mutants isolated in experiment 90, plate number 1, well B5, the *E. coli* strain is 90-1B5, the mutant gene is *luc90-1B5*, and the mutated luciferase is *Luc90-1B5*.

As defined herein, a “thermostable” enzyme, e.g., a luciferase, or an enzyme which has “thermostability”, is an enzyme which under certain conditions, e.g., at certain temperature, in aqueous solution and/or for certain periods of time, has an increased retention of activity relative to a reference enzyme. For example, for a thermostable luciferase, a reference luciferase may be native wild-type luciferase or recombinant wild-type luciferase. Preferably, for beetle luciferases, the activity is luminescence under conditions of saturation with luciferin and ATP. One measure of thermostability of an enzyme is the half-life of the enzyme in an aqueous solution (the time over which 50% of the activity is lost) at a stated temperature.

The invention further encompasses expression vectors and other genetic constructs containing the mutant luciferases, as well as hosts, bacterial and otherwise, transformed to express the mutant luciferases. The invention is also drawn to compositions and kits which contain the novel luciferases, and use of these luciferases in any methodology where luciferases are employed.

Various means of random mutagenesis were applied to a luciferase gene (nucleotide sequence), most particularly gene synthesis using an error-prone polymerase, to create libraries of modified luciferase genes. This library was expressed in colonies of *E. coli* and visually screened for efficient luminescence to select a subset library of modified luciferases. Lysates of these *E. coli* strains were then made, and quantitatively measured for luciferase activity and thermostability. From this, a smaller subset of modified luciferases was chosen, and the selected mutations were combined to make composite modified luciferases. New libraries were made from the composite modified luciferases by random mutagenesis and the process was repeated. The luciferases with the best overall performance were selected after several cycles of this process.

Methods of producing improved luciferases include directed evolution using a polynucleotide sequence encoding a first beetle luciferase as a starting (parent) sequence, to produce a polynucleotide sequence encoding a second luciferase with increased thermostability, compared to the first luciferase, while maintaining other characteristics of the enzymes. A cDNA designated *lucPpe2* encodes a firefly luciferase derived from *Photuris pennsylvanica* that displays increased thermostability as compared to the widely utilized luciferase



designated *LucPpy* from *Photinus pyralis*. The cDNA encoding *LucPpe2* was isolated, sequenced and cloned (see Leach et al., 1997). A mutant of this gene encodes a first luciferase *LucPpe2* [T249M]. However, the methods of the invention are not limited to use with a polynucleotide sequence encoding a beetle  
5 luciferase, i.e., the methods of the invention may be employed with a polynucleotide sequence encoding other enzymes.

In an embodiment of a mutant luciferase, the amino acid sequence is that of *LucPpe2* shown in Figure 45 with the exception that at residue 249 there is a M (designated T249M) rather than the T reported by Leach et al. The underlined  
10 residue (249) shows mutation from T to M. This enzyme produced approximately 5-fold more light *in vivo* when expressed in *E. coli*.

Diluted extracts of recombinant *E. coli* that expressed mutant luciferases made by the methods of the invention were simultaneously screened for a plurality of characteristics including light intensity, signal stability, substrate  
15 utilization ( $K_m$ ), and thermostability. A fully automated robotic system was used to screen large numbers of mutants in each generation of the evolution. After several cycles of mutagenesis and screening, thereby creating mutant libraries of luciferases, an increased thermostability compared to *LucPpe2* [T249M] of about 35°C was achieved for clone Luc90-1B5 which also essentially maintained  
20 enzymatic activity (there was only negligible loss in activity of 5%) when kept in aqueous solution over 2 hours at 50°C, 5 hours at 65°C, or over 6 weeks at 22°C.

Mutant luciferases of the present invention display increased thermostability for at least 2 hours at 50°C, preferably at least 5 hours at 50°C, and in the range of at least 2 hours, preferably at least 24 hours, and more  
25 preferably at least 50 hours, at temperatures including 50°C, 60°C, and/or at temperatures up to 65°C. In particular, the present invention comprises thermostable mutant luciferases which, when solubilized in a suitable aqueous solution, have a thermostability greater than about 2 hours at about 50°C, more preferably greater than about 10 hours at 50°C, and more preferably still greater  
30 than 5 hours at 50°C. The present invention also comprises mutant luciferases which, when solubilized in a suitable aqueous solution, have a thermostability greater than about 2 hours, more preferably at least 5 hours, even more preferably greater than about 10 hours, and even more preferably still greater

than about 24 hours, at about 60°C. The present invention further comprises mutant luciferases which when solubilized in a suitable aqueous solution have a thermostability greater than about 3 months at about 22°C, and more preferably a thermostability of at least 6 months at 22°C. An embodiment of the invention is a luciferase mutant having thermostability at 65°C, wherein a loss of activity of about 5-6% was found after 6 hours (equivalent to a half-life of 2 days). The half-lives of enzymes from the most stable clones of the present invention, extrapolated from data showing small relative changes, is greater than 2 days at 65°C (corresponding to 6% loss over 6 hours), and about 2 years at 22°C (corresponding to 5% loss over 9 weeks).

In particular, the invention comprises luciferase enzymes with embodiments of amino acid sequences disclosed herein (e.g., mutant luciferases designated Luc49-7C6, Luc78-0B10; Luc90-1B5, Luc133-1B2, and Luc146-1H2, as well as all other beetle luciferases that have thermostability as measured in half-lives of at least 2 hours at 50°C. The invention also comprises mutated polynucleotide sequences encoding luciferase enzymes containing any single mutation or any combination of mutations of the type which convert an amino acid of the reference beetle luciferase into a consensus amino acid. Conserved amino acids are defined as those that occur at a particular position in all sequences in a given set of related enzymes. Consensus amino acids are defined as those that occur at a particular position in more than 50% of the sequences in a given set of enzymes. An example is the set of beetle luciferase sequences shown in Figure 19, excluding *LucPpe2*.

Nucleotide sequences encoding beetle luciferases are aligned in Figure 19. Eleven sequences found in nature in various genera, and species within genera, are aligned, including *lucPpe2*. There are at least three mutations present in each mutant luciferase that show increased thermostability. In general, mutations are not of a conserved amino acid residue. The mutations in the mutant luciferases are indicated in Figures 22-47 by underlining.

The invention also provides methods to prepare enzymes having one or more desired properties, e.g., resistance to inhibition by a substrate analog of the enzyme or enhanced enzymological properties. The method comprises selecting at least one isolated polynucleotide sequence encoding an enzyme with the

desired property, e.g., an enzymological property, from a first population of mutated polynucleotide sequences. The selected, isolated polynucleotide sequence is then mutated to yield a second population of mutated polynucleotide sequences. Preferably, a mixture of selected isolated polynucleotide sequences are mutated to yield a second population of mutated polynucleotide sequences. The process may be repeated until a further polynucleotide sequence is obtained, e.g., selected and/or isolated, which further polynucleotide sequence encodes an enzyme which has at least one of the desired properties. As used herein, the terms "isolated and/or "purified" refer to *in vitro* isolation of a RNA, DNA or polypeptide molecule from its natural cellular environment, and from association with other components of the cell, such as nucleic acid or polypeptide, e.g., so that it can be sequenced, replicated, and/or expressed.

#### **Brief Description of the Drawings**

Figure 1 is a graphical representation of thermostability at 37°C of *LucPpe2*[T249M]; *Luc39-5B10*; and *Luc49-7C6*, normalized to  $t = 0$  [the X-axis is time in minutes; the Y-axis is % remaining activity; and "t" is time].

Figure 2 is a graphical representation of the remaining activity of *Luc49-7C6* and *Luc78-0B10* at 50°C normalized to a  $t = 0$  reading [the X-axis is time in hours; the Y-axis is % remaining activity; and t is time].

Figure 3 is a graphical representation of the luminescence produced by *Luc49-7C6* and *Luc78-0B10* at 60°C normalized to  $t = 0$  [the X-axis is time in hours; the Y-axis is % remaining activity; and t is time].

Figure 4 is a graphical representation of thermostability of luciferases, *LucPpe2*[T249M]; *Luc49-7C6*; and *Luc78-0B10* thermostability at 22°C [the X-axis is time in days; the Y-axis is normalized light units].

Figure 5 is a graphical representation of the observed log luminescence produced by (Y) *Luc78-0B10* compared to log luminescence predicted by the regression equation  $Y = 0.0043X + 10.91$ ; the half life of the enzyme is calculated as 144 hours (6 days) [the X-axis is time in hours; the Y-axis is log luminescence].

Figure 6 is a graphical representation of the observed log luminescence produced by *Luc78-0B10* at 60°C compared to the log luminescence calculated by the regression equation  $Y = 0.154X + 10.86$ ; the half life of the enzyme is

calculated as 38 hours (1.58 days) [the X-axis is time in hours; the Y-axis is log luminescence].

Figure 7 is a graphical representation of the observed log luminescence produced by Luc49-7C6 at 50°C compared to log luminescence predicted by the regression equation  $Y = -0.0059X + 8.757$ ; the half-life of the enzyme is  
5 calculated as 100.5 hours (4.2 days) [the X-axis is time in hours; the Y-axis is log luminescence].

Figure 8 is a graphical representation of the observed log luminescence produced by Luc49-7C6 at 60°C compared to the log luminescence calculated by  
10 the regression equation  $Y = -0.169X + 8.647$ ; the calculated half-life of the enzymes is 2.9 hours (the X-axis is time in hours; the Y-axis is log luminescence).

Figure 9 is a graphical representation of the observed log luminescence produced by Luc78-0B10 at 22°C compared to a predicted log luminescence, the  
15 half-life of the enzyme is 109 days [the X-axis is time in days; the Y-axis is log luminescence].

Figure 10 is a graphical representation of the observed luciferase log luminescence produced by Luc49-7C6 at 22°C compared to a predicted log luminescence; the half-life of the enzyme is 64 days [the X-axis is time in days;  
20 the Y-axis is log luminescence].

Figure 11 is a graphical representation of the observed log luminescence produced by luciferase Luc49-7C6 at 37°C compared to predicted log luminescence [the X-axis is time in minutes; the Y-axis is log luminescence].

Figure 12 is a graphical representation of the observed log luminescence  
25 produced by luciferase LucPpe2 [T249M] at 22°C compared to predicted log luminescence [the X-axis is time in days; the Y-axis is log luminescence].

Figure 13 is a graphical representation of the observed log luminescence produced by luciferase LucPpe2 [T249M] at 37°C compared to predicted log luminescence [X-axis is time in minutes; Y-axis is log luminescence].

30 Figure 14 is a flow chart showing steps for an assay of *in vivo* and *in vitro* luciferase luminescence (Li); enzyme stability ( $\tau$ ); assay kinetics (S); and substrate binding (Km).

Figure 15 is a schematic representation of a table top layout robot.

Figure 16A is a graphical representation of luciferase mutant Luc90-1B5 luminescence measured at 65°C, pH 6.5 (the X-axis is time in hours; the Y-axis is % luminescence).

Figure 16B is a graphical representation of luciferase mutant Luc90-1B5  
5 luminescence at 22°C, pH 6.5 (the X-axis is time in days; the Y-axis is % luminescence).

Figure 17 is a diagram showing the evolutionary relationships among beetle luciferases based on amino acid sequences.

Figure 18A is a representation of the secondary structures of beetle  
10 luciferase enzymes (helices are symbolized by cylinders, sheets by collections of arrows, loops connect helices with sheets).

Figure 18B shows the amino acids (tertiary structures) of the *LucPpe2* luciferase, wherein small spirals correspond to cylinders of Figure 18A.

Figure 18C shows that the general beetle architecture matches (is  
15 superimposed on) that of Luc90-1B5.

Figure 19A presents alignment of the amino acid sequence (SEQ ID Nos:27-37) for luciferases from various beetle species (Lcr, Lla, Lmi, Pmi, Ppy, Lno, Ppe1, Phg, GR, YG, Ppe2, respectively) and luciferases of the present invention (Luc49-7C6; Luc78-0B10; Luc90-1B5, Luc133-1B2; and Luc146-  
20 1H2, SEQ ID Nos. 14, 19, 24, 44, and 45, respectively); the sequences are aligned, spaces where sequences cannot be aligned are shown by dots (e.g., . . . ); only amino acids that differ in the luciferases of the present invention from those of some beetle species are shown, not the full sequences. "Cons" is a sequence showing conserved amino acids by single letters, and indicates non-conserved  
25 amino acids by "-".

Figure 19B presents alignment of the amino acid sequence (SEQ ID Nos:27-37) for luciferases from various beetle species (Lcr, Lla, Lmi, Pmi, Ppy, Lno, Ppe1, Phg, GR, YG, Ppe2) and luciferases of the present invention (Luc30-4B02 and Luc81-6G01, SEQ ID Nos. 47 and 26, respectively); the sequences are  
30 aligned, spaces where sequences cannot be aligned are shown by dots (e.g., . . . ); amino acids that differ in the luciferases of the present invention from those of some beetle species are shown in bold.

Figure 19C presents alignment of the amino acid sequence (SEQ ID NOs:27-34 and 36-37) for luciferases from various beetle species (Lcr, Lla, Lmi, Pmi, Ppy, Lno, Ppe1, Phg, YG, Ppe2, Ppl); the sequences are aligned, spaces where sequences cannot be aligned are shown by dots (e.g., . . . ); in the line  
5 beneath YG, X indicates positions in YG where mutations could yield a consensus amino acid; O indicates positions in YG where mutations could not yield a consensus amino acid.

Figure 20 is the 7216 bp *Ppe2* vector map in a pRAM backbone.

Figure 21 is a bar graph comparing luminescence as expressed in  
10 recombinant colonies of *E. coli*; the colonies differ in the identity of the luciferase encoding vector (Luc+; Luc90-1B5; Luc78-1B10; Luc49-7C6; *LucPpe2* [T249M] and *LucPpe2*); in the recombinant colony shown in the Y-axis [the X-axis is normalized light units].

Figure 22 is a nucleotide (DNA) sequence (SEQ ID NO:1) encoding  
15 mutant luciferase enzyme Luc49-7C6; mutations are indicated by underlining.

Figure 23 is a nucleotide (DNA) sequence (SEQ ID NO:2) encoding mutant luciferase enzyme Luc49-6C10, mutations are indicated by underlining.

Figure 24 is a nucleotide (DNA) sequence (SEQ ID NO:3) encoding a mutant luciferase enzyme Luc49-0G12; mutations are indicated by underlining.

20 Figure 25 is a nucleotide (DNA) sequence (SEQ ID NO:4) encoding a mutant luciferase enzyme Luc49-7A5; mutations are indicated by underlining.

Figure 26 is a nucleotide (DNA) sequence (SEQ ID NO:5) encoding a mutant luciferase enzyme Luc49-4G11; mutations are indicated by underlining.

25 Figure 27 is an amino acid sequence (SEQ ID NO:14) of the mutant luciferase designated Luc49-7C6; mutations are indicated by underlining.

Figure 28 is an amino acid sequence (SEQ ID NO:15) of mutant luciferase enzyme Luc49-6C10; mutations are indicated by underlining.

Figure 29 is an amino acid sequence (SEQ ID NO:16) of mutant luciferase enzyme Luc49-0G12; mutations are indicated by underlining.

30 Figure 30 is an amino acid sequence (SEQ ID NO:17) of mutant luciferase enzyme Luc49-7A5; mutations are indicated by underlining.

Figure 31 is an amino acid sequence (SEQ ID NO:18) of mutant luciferase enzyme Luc49-4G11; mutations are indicated by underlining.

Figure 32 is a nucleotide (DNA) sequence (SEQ ID NO:6) encoding mutant luciferase enzyme Luc78-0B10; mutations are indicated by underlining.

Figure 33 is a nucleotide (DNA) sequence (SEQ ID NO:7) encoding mutant luciferase enzyme Luc78-0G8; mutations are indicated by underlining;

5 X's signify unknown identities of nucleotides at certain positions.

Figure 34 is a nucleotide (DNA) sequence (SEQ ID NO:8) encoding mutant luciferase enzyme Luc78-1E1; mutations are by underlining; X's signify that the identity of a nucleotide at a position is unknown.

Figure 35 is a nucleotide (DNA) sequence (SEQ ID NO:9) encoding a  
10 mutant luciferase Luc78-2B4; underlined nucleotides are mutations; X's signify unknown identities of nucleotides at certain positions.

Figure 36 is an amino acid sequence (SEQ ID NO:19) of the mutant luciferase Luc78-0B10; underlined amino acids are mutations.

Figure 37 is an amino acid sequence (SEQ ID NO:20) of the mutant  
15 luciferase enzyme Luc78-0G8; underlined amino acids are mutations; X's signify unknown amino acids at a position.

Figure 38 is an amino acid sequence (SEQ ID NO:21) for mutant luciferase enzyme Luc78-1E1; underlined amino acids are mutations; X's signify an unknown amino acid at a position.

20 Figure 39 is an amino acid sequence (SEQ ID NO:22) for mutant luciferase enzyme Luc78-2B4; underlined amino acids are mutations; X's signify an unknown amino acid at a position.

Figure 40 is a nucleotide (DNA) sequence (SEQ ID NO:10) for encoding a mutant luciferase enzyme Luc85-4F12; underlined nucleotides are mutations;  
25 X's signify an unknown amino acid at that position.

Figure 41 is an amino acid listing (SEQ ID NO:23) for a mutant luciferase enzyme Luc85-4F12; underlined amino acids are mutations; X's signify an unknown amino acid at that position.

Figure 42 is a nucleotide (DNA) sequence (SEQ ID NO:11) encoding  
30 mutant luciferase enzyme Luc90-1B5; underlined nucleotides are mutations.

Figure 43 is an amino acid sequence (SEQ ID NO:24) for the mutant luciferase designated Luc90-1B5; underlined amino acids are mutated positions.

Figure 44 is a nucleotide (DNA) sequence (SEQ ID NO:12) encoding luciferase enzyme *LucPpe2* [T249M].

Figure 45 is an amino acid sequence (SEQ ID NO:25) for *LucPpe2* [T249M]; the underlined amino acid is a mutation from Thr to Met at residue  
5 249.

Figure 46 is an amino acid sequence (SEQ ID NO:26) for luciferase enzyme *LucPp181-6G1*; underlined amino acids are mutations from a starting sequence; X shows ambiguity.

Figure 47 is a nucleotide (DNA) sequence (SEQ ID NO:13) encoding  
10 luciferase enzyme *Luc81-6G1*; underlined nucleotides are mutations.

Figure 48 is a graphical representation of mutant luciferases *Luc49-7C6* and *Luc78-0B10* luminescence at 60°C normalized to  $t = 0$  [the X-axis is time in hours, the Y-axis is log normalized luminescence].

Figure 49 is a graphical representation of luciferases *LucPpe2* [T249M],  
15 *Luc49-7C6*, and *Luc78-0B10*, thermostability at 4°C, normalized to initial values [the X-axis is time in days; Y is log normalized light units].

Figure 50 is a graphical representation of mutant luciferases *Luc49-7C6* and *Luc78-0B10* luminescence at 50°C normalized to  $t = 0$  [the X-axis is time in hours; the Y-axis is log luminescence].

20 Figure 51 is a graphical representation of mutant luciferases *Luc49-7C6* and *Luc78-0B10* luminescence at 50°C normalized at  $t = 0$ .

Figure 52 is a graphical representation of mutant luciferases *Luc49-7C6* and *Luc78-0B10* luminescence at 60°C normalized to  $t = 0$  [the X-axis is time in hours; the Y-axis is luminescence].

25 Figure 53 is a graphical representation of luciferases *LucPpe2* [T249M], *Luc49-7C6*, and *Luc78-0B10* thermostability at 22°C [the X-axis is time in days; the Y-axis is log luminescence].

Figure 54A is a graphical representation of luminescence of *Luc90-1B5*; *Luc133-1B2*; and *Luc146-1H2*, at pH 4.5 and 48°C, normalized to  $t = 0$ .

30 Figure 54B is a graphical representation of the half-life of *Luc90-1B5*; *Luc133-1B2*; and *Luc146-1H2*, at pH 4.5 and 48°C. The half-life of *Luc90-1B5* under these conditions is about 3 minutes, *Luc133-1B2* about 20 minutes, and *Luc146-1H2* about 62 minutes.



Figure 55 is a nucleotide (DNA) sequence (SEQ ID NO:42) encoding a luciferase enzyme Luc133-1B2; mutations are indicated by underlining.

Figure 56 is a nucleotide (DNA) sequence (SEQ ID NO:43) encoding a luciferase enzyme Luc146-1H2; mutations are indicated by underlining.

5        Figure 57 is an amino acid sequence (SEQ ID NO:44) of mutant luciferase Luc133-1B2; mutations are indicated by underlining.

Figure 58 is an amino acid sequence (SEQ ID NO:45) of mutant luciferase Luc146-1H2; mutations are indicated by underlining.

Figure 59 is a graphical representation of the signal kinetics of clones  
10    Luc49-7C6; Luc78-0B10; Luc90-1B5; Luc133-1B2; and Luc146-1H2 at pH 7.8 at room temperature.

Figure 60 is a graphical representation of the normalized luminescence at 50°C pH 7.8 of Luc49-7C6; Luc78-0B10; Luc90-1B5; Luc133-1B2; and Luc146-1H2; from t = 0 to about 8 hours.

15        Figure 61 is a graphical representation of the resistance of selected luciferases to a substrate inhibitor. The data is presented as the log of the luminescence versus time for Luc90-1B5; Luc133-1B5; and Luc146-1H2.

Figure 62 is a graphical representation of the log of luminescence over time at 22°C, pH 6.5 for Luc90-1B5 and LucPpe2[T249M].

20        Figure 63 is a graphical representation of thermostability of selected mutant luciferases and Luc*PpIYG* at room temperature in aqueous solution containing 1% Triton X-100.

Figures 64 is a graphical representations of the sustained luminescence activity (expressed as luminescence/O.D.) over time for certain luciferases.

25        Figure 65 is a nucleotide (DNA) sequence (SEQ ID NO:46) encoding a luciferase enzyme Luc81-0B11; mutations are indicated by underlining.

Figure 66 is an amino acid sequence of mutant luciferase Luc81-0B11; mutations are indicated by underlining.

**Detailed Description of the Invention**

The invention relates to enzymes, e.g., beetle luciferases, that are created by mutations made in the encoding genes, generally by recursive mutagenesis, which mutated enzymes have one or more desired properties, for example,

5 increases thermostability, increased resistance to inhibitors, and/or enhanced enzymological properties, relative to a reference enzyme, e.g., the wild-type enzyme. The polynucleotide sequence which encodes an enzyme of the invention comprises mutations that encode a plurality of amino acid substitutions relative to the polynucleotide sequence encoding the enzyme from which the

10 enzyme of the invention was derived. For example, the invention relates to enzymes, e.g., luciferases, that are thermostable. The increased thermostability allows storage of enzymes such as luciferases without altering its activity, and improves reproducibility and accuracy of assays using the mutated luciferases. Thus, one embodiment of the invention comprises isolated polynucleotide

15 sequences (cDNAs) which encode mutant luciferases with increased thermostability, vectors containing the polynucleotide sequences, and hosts transformed to express the polynucleotide sequences. Table 1 shows results of about 250 clones and characteristics of the luciferases from the clones including thermostability. The invention also encompasses the use of the mutant

20 luciferases in any application where luciferases are conventionally utilized, and kits useful for some of the applications.

Unexpectedly, beetle luciferases with the sought after improved thermostability were achieved in the present invention through a process of recursive mutagenesis and selection (sometimes referred to as “directed

25 evolution”). A strategy of recursive mutagenesis and selection is an aspect of the present invention, in particular the use of multi-parameter automated screens. Thus, instead of screening for only a single attribute such as thermostability, simultaneous screening was done for additional characteristics of enzyme activity and efficiency. By this method, one property is less likely to “evolve” at

30 the expense of another, resulting in increased thermostability, but decreased activity, for example.

Table 1 presents examples of parameter values ( $L_i$ ,  $\tau$ ,  $K_m$  and  $S$ , see below) derived from experiments using different luciferases as starting (parent)

sequences. The subtitles refer to designations of the temperature at which the enzyme stability was measured and the starting luciferase, e.g., Luc39-5B10 at 51°C and so forth. All parameters in each experiment are recorded as relative values to the respective starting sequence, e.g., the parameter values for the starting sequence in any experiment equals "1." (See Example 2 herein for definitions.)

Thermostability has evolved in nature for various enzymes, as evidenced by thermostable isozymes found in thermophilic bacteria. Natural evolution works by a process of random mutagenesis (base substitutions, gene deletions, gene insertions), followed by selection of those mutants with improved characteristics. The process is recursive over time. Although the existence of thermostable enzymes in nature suggests that thermostability can be achieved through mutagenesis on an evolutionary scale, the feasibility of achieving a given level of thermostability for a particular class of enzymes by using short term laboratory methods was unpredictable. The natural process of evolution, which generally involves extremely large populations and many millions of generations and genes, by mutation and selection cannot be used to predict the capabilities of a modern laboratory to produce improved genes by directed evolution until such mutants are produced.

After such success, because the overall three-dimensional structure of all beetle luciferases are quite similar, having shown it possible for one member of this class makes it predictable that high thermostability can be achieved for other beetle luciferases by similar methods. Figure 17 shows an evolutionary relationship among beetles luciferases, all of which have a similar overall architecture. The structural class to which the beetle luciferases belong is determined by the secondary structure (e.g. helices are symbolized by cylinders, sheets by collections of arrows, loops connect helices with sheets (Figure 18A). Figure 18B shows the amino acids of the *LucPpe2* luciferase wherein small spirals correspond to cylinders of Figure 18A; Figure 18C shows that the general beetle architecture matches (is superimposed on) that of *LucPpe2*. This is support for the expectation that the methods of the present invention can be generalized to all beetles luciferases.

Enzymes belong to different structural classes based on the three-dimensional arrangement of secondary elements such as helices, sheets, and loops. Thermostability is determined by how efficiently the secondary elements are packed together into a three-dimensional structure. For each structural class, there also exists a theoretical limit for thermostability. All beetle luciferases belong to a common structural class as evident by their common ancestry (Figure 17), homologous amino acid sequences, and common catalytic mechanisms.

The application of a limited number of amino acid substitutions by mutagenesis is unlikely to significantly affect the overall three-dimensional architecture (i.e., the structural class for mutant luciferases is not expected to change.) Because the theoretical limit for thermostability for any structural class is not known, the potential thermostability of beetle luciferases was not known until demonstrations of the present invention.

*A priori* difficulties in achieving the goals of the present invention included:

1. The types of mutations which can be made by laboratory methods are limited.

- i) By random point mutation (e.g. by error-prone PCR), more than one base change per codon is rare. Thus, most potential amino acid changes are rare.
- ii) Other types of random genetic changes are difficult to achieve for areas greater than 100 bp (e.g., random gene deletions or insertions).
2. The number of possible luciferase mutants that can be screened is limited.
- i) Based on sequence comparisons of natural luciferases, ignoring deletions and insertions, more than  $10^{189}$  functional enzyme sequences may be possible.
- ii) If 100,000 clones could be screened per day, it would require more than  $10^{179}$  centuries to screen all possible mutants assuming same mutant was never screened twice (actual screening rate for the present invention was less than 5000 per day).

3. The probability of finding functional improvement requiring cooperative mutations is rare (the probability of finding a specific cooperative pair is 1 out of  $10^8$  clones).

Thus, even if the theoretical limits of thermostability were known,  
5 because only a very small number of the possible luciferase mutants can be screened, the *a priori* probability of finding such a thermostable enzyme was low.

However, the present invention now shows that it is possible and feasible to create novel beetle luciferases having high thermostability.

- 10 a) The approximately 250 mutants produced by methods of the present invention wherein the initial sequence was from *lucPpe2* or *lucPpIYG* demonstrate that it is possible and feasible for at least one member of this enzyme class to achieve high thermostability.
- 15 b) Any beetle luciferase can be improved by similar means because the luciferases belong to the same structural class.
- i) Because all beetle luciferases belong to the same structural class, they also share in the same pool of potentially stabilizing mutations (this conclusion is supported by  
20 observation that a high percentage of the stabilizing mutations found in the clones of the present invention were conversions to "consensus amino acids" in other beetle luciferases that is, amino acids that appear in the majority of beetle luciferase sequences (see Figure 19).
- 25 ii) Similar results were achieved using beetle luciferase, consisting largely of a different amino acid sequence, from the luminous beetle *Pyrophorus plagiophthalmus* (*LucPpIYG*). The wild-type *lucPpIYG* has 48% nucleotide sequence identity to the wild type *lucPpe2*.
- 30 The *LucPpIYG* mutants were subjected to fewer cycles of directed evolution than the *LucPpe2* mutants described herein. Also, in some instances, mutants were selected with less emphasis placed on their relative thermostability.

The most stable clone resulting from this evolution (Luc80-5E5) has a half-life of roughly 3.8 hours at 50°C in solution.

To compensate for a statistical effect caused by the large number of deleterious random mutations expected relative to the beneficial mutations, methods were employed to maximize assay precision and to re-screen previously selected mutations in new permutations. Among the methods for maximizing assay precision were closely controlling culture conditions by using specialized media, reducing growth rates, controlling heat transfer, and analyzing parameters from mid-logarithmic phase growth of the culture. The robotic processes maximized for precision include controlling mixing, heat transfers, and evaporation of samples in the robotic screening process; and normalizing data to spatially distributed control samples. New permutations of the selected mutations were created by a method of DNA shuffling using proof-reading polymerases.

The difficulty in predicting the outcome of the recursive process is exemplified by the variable success with the other characteristics of luciferase that were also selected for. Although the primary focus was on the enzyme thermostability, selection for mutants producing brighter luminescence, with more efficient substrate utilization, and an extended luminescence signal was also attempted. The definitions are given by equations herewith. The selection process was determined by changes relative to the parent clones for each iteration of the recursive process. The amount of the change was whatever was observed during the screening process. The expression of luciferase in *E. coli* was relatively inefficient, for LucPpe2, compared to Luc+. Other luciferases varied (see Figure 21).

To improve the overall efficiency of substrate utilization, reduction in the composite apparent utilization constant (i.e.,  $K_m[\text{ATP} + \text{luciferin}]$ ) for both luciferin and ATP was sought. Although there was an unexpected systematic change in each utilization constant ( $K_m[\text{ATP}]$ ,  $K_m[\text{luciferin}]$ ), there was little overall change. Finally, the luminescence signal could only be moderately affected without substantially reducing enzyme efficiency. Thus, although the

enzyme thermostability was greatly increased by methods of the present invention, other characteristics of the enzyme were much less affected.

Figures 1-13, 16, 48-53, 60 and 62 present measurements of thermostability of mutant luciferases. Figures 48-53 present other results of the mutant luciferases. Compositions of the invention include luciferases having greater than the natural level of thermostability. Each mutant luciferase is novel, because its individual characteristics have not been reported. Specific luciferases are known by both their protein and gene sequences. Many other luciferases were isolated that have increased, high thermostability, but whose sequences are not known. These luciferases were identified during the directed evolution process, and were recognized as distinct by their enzymological characteristics. The mutant luciferases of the present invention, e.g., Luc90-1B5, can display remarkable and heretofore unrealized thermostability at temperatures ranging from 22°C to at least as high as 60°C.

Other aspects of the invention include methods that incorporate the thermostable luciferases, specifically beetle luciferases having high thermostability, as well as methods to prepare an enzyme, including a luciferase, having one or more desired properties, e.g., resistance to inhibition by a substrate inhibitor, or enhanced enzymological properties. Thus, the invention also provides a method to prepare an enzyme which has at least one enhanced enzymological property. From a population of polynucleotide sequences encoding the enzyme which is derived from a first polynucleotide sequence encoding the enzyme which is subject to mutation, at least one polynucleotide sequence encoding an enzyme which has the enhanced enzymological activity is selected and isolated. In one embodiment, oligonucleotide-mediated mutagenesis is then employed to introduce at least one codon which encodes a consensus amino acid to at least one of the selected, isolated polynucleotide sequences encoding the enzymes to yield a further polynucleotide sequence encoding the enzyme and having the codon which encodes the consensus amino acid, wherein the codon which is introduced is not present in the first polynucleotide sequence.

### Production of Luciferases of the Present Invention

The method of making luciferases with increased thermostability is recursive mutagenesis followed by selection. Embodiments of the highly thermostable mutant luciferases of the invention were generated by a reiterative  
5 process of random point mutations beginning with a source nucleotide sequence, e.g., the *lucPpe2* [T249M] cDNA. Recombination mutagenesis is a part of the mutagenesis process, along with point mutagenesis. Both recombination mutagenesis and point mutagenesis are performed recursively. Because the mutation process causes recombination of individual mutants in a fashion similar  
10 to the recombination of genetic elements during sexual reproduction, the process is sometimes referred to as the sexual polymerase chain reaction (sPCR). See, for instance, Stemmer, U.S. Patent No. 5,605,793, issued February 25, 1997.

Taking the *lucPpe2* cDNA sequence as a starting point, the gene was mutated to yield mutant luciferases which are far more thermostable. A single  
15 point mutation to the *lucPpe2* sequence yielded the luciferase whose sequence is depicted as T249M. This mutant is approximately 5 times brighter *in vivo* than that of *lucPpe2*, it was utilized as a template for further mutation. It was also used a baseline for measuring the thermostability of the other mutant luciferases described herein.

### Embodiments Of Sequences Of Luciferases Of The Present Invention

Figure 45 shows the amino acid sequence of the *LucPpe2* luciferase (T249M). The sequence contains a single mutation at position 249 from T to M (underlined) which distinguishes it from the sequence reported by Leach et al. (1997). This luciferase has a spectral maximum of 552 nm, which is yellow  
25 shifted from that of the luciferase of Leach et al. This mutant was selected for use as an original template in some of the Examples because it is approximately 5 times brighter *in vivo*, than the form reported by Leach et al. which allowed for more efficient screening by the assay. These sequences show changes from the starting sequence (T249M) by underlining. Note that "x" in the sequence  
30 denotes an ambiguity in the sequence.



Directed Evolution, A Recursive Process

Directed evolution is a recursive process of creating diversity through mutagenesis and screening for desired changes. For enzymological properties that result from the cumulative action of multiple amino acids, directed evolution  
5 provides a means to alter these properties. Each step of the process typically produces small changes in enzyme function, but the cumulative effect of many rounds of this process can lead to substantial overall change.

The characteristic, "thermostability" is a candidate for directed evolution because it is determined by the combined action of many of the amino acids  
10 making up the enzyme structure. Luminescence output and efficiency of substrate binding of the modified luciferase were also screened. This was to ensure that changes in thermostability did not also produce undesirable changes in other important enzymological properties.

Because the frequency of deleterious mutations is much greater than  
15 useful mutations, it is likely that undesirable clones are selected in each screen within the precision limits of the present invention. To compensate for this, the screening strategy incorporated multiple re-screens of the initially selected mutations. However, before re-screening, the selected mutations were "shuffled" to create a library of random intragenetic recombinations. This process allows  
20 beneficial mutations among different clones to be recombined together into fewer common coding sequences, and unlinks deleterious mutations to be segregated and omitted. Thus, although essentially the same set of selected mutations was screened again, they were screened under different permutations as a result of the recombination or shuffling.

25 Although results of each step of the evolutionary process were assayed by quantitative measurements, these measurements were mutually made in cell lysates rather than in purified enzymes. Furthermore, each step only measured changes in enzyme performance relative to the prior step, so global changes in enzyme function were difficult to judge.

30 Table 1 summarizes the characteristics of various clones obtained using the methods of the invention.

**Table 1**

Control is Luc39-5B10 at 51°C

	Experiment	Clone ID	Luminescence (Li)	Enzyme stability (tau)	Substrate binding (Km)	Signal stability (S)
5	40	0a7	1.04	4.5	0.78	1
	40	5h4	1.29	1.61	1.16	0.953
	40	0c2	1.13	1.54	0.91	0.998
	40	5g4	1	1.4	0.85	1
	40	6d3	1.02	1.37	0.79	1
10	40	1g4	1.06	1.28	0.77	0.985
	40	1d4	1.69	1.23	0.73	1
	40	0h9	1.26	1.21	0.63	0.998
	40	2f6	3	1.07	0.49	0.981
	40	7d6	3.09	1.058	1.09	1.013
15	40	5a7	4.3	1.025	0.93	1.008
	40	4c8	1	1	0.33	1.004

	Experiment	Clone ID	Li	tau	Km	S
20	41	7h7	0.73	2.4	2.1	0.995
	41	5a5	0.77	1.93	2.7	1.002
	41	2c12	1.06	1.7	0.91	1.003
	41	6e5-	1.16	1.62	1.53	0.997
	41	4e5-	1.08	1.37	1.4	1.004
	41	6g7	1.3	1.27	1.39	0.999
25	41	1h4	1.36	1.24	0.56	0.994
	41	0c11	4.1	1.23	1.24	0.996
	41	2h9	5.3	1.01	0.83	0.986
	42	6b10	0.97	3.6	0.97	0.997
	42	1c3	0.91	2.1	0.6	0.998
30	42	7h9	0.8	1.8	0.8	0.982
	42	6b2	0.77	1.72	0.8	0.978
	42	6d6	0.83	1.7	0.733	0.975
	42	4e10-	0.77	1.63	1.8	0.954
	42	1b5	0.83	1.41	1.05	0.955
35	42	6e6-	0.71	1.16	0.89	0.955

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42	3a9	0.85	1.3	0.86	0.997
42	6b6	2.7	1.3	0.91	1.02
42	6e9-	1.5	1.27	0.98	1.01
42	3h11	1.73	1.21	0.63	0.985
42	1a2	1.11	1.17	0.77	1.005
42	3f7	0.49	1.16	1.13	0.944
42	1a4	2	1.01	0.76	0.996

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Control is Luc40-0A7 at 54°C

Experiment	Clone ID	Li	tau	Km	S
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46	2h3	0.86	6.4	0.37	0.96
46	4a9	0.67	5.7	0.66	0.997
46	2g4	0.65	5.3	0.78	0.96
46	5d12	0.94	4.9	0.94	1.002
46	1h11	1.02	4.8	0.84	0.998
46	5a10	1.23	4.4	0.81	0.9842
46	0a8	1.35	4.3	0.89	1
46	4d3	0.51	3.6	0.65	0.975
46	2a3	1.17	2.9	0.57	0.988
46	3b11	1.39	2.5	0.63	1.02
46	7g12	1.49	2.5	0.91	1.02
46	0g9	1.86	2.25	0.5	0.998
46	7h8	1.07	1.36	0.52	0.99
46	1g8	0.3	1.31	0.72	0.92
46	1d3	1.74	1.13	1.02	1.001
46	0c3	1.68	1.01	0.74	1.01
46	5c11	0.82	1.01	0.6	0.95

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Control is Luc46-2H3 at 54°C

Experiment	Clone ID	Li	tau	Km	S
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49	6c10	0.57	2.2	0.98	1
49	7c6	1.12	1.9	0.93	1.01
49	0g12	1	1.58	0.69	1.08
49	7a5	1.08	1.44	1.1	0.99

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49	1f6	0.66	1.13	1.04	1.006
49	0b5	0.76	1.07	1.03	0.98
49	4a3	0.94	1.06	0.77	1

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Control is Luc49-7C6 at 56°C

Experiment      Clone                      Li            tau            Km            S  
                         ID

56	2d12	0.97	2.9	0.29	1.006
56	5g10	1.01	2.77	0.64	1.007
56	3d5	1.32	2.25	1.85	1.0

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Experiment      Clone                      Li            tau            Km            S  
                         ID

57	3d1	1.06	2.9	1.05	1.02
57	6g12	1	2.7	0.87	1.004
57	4c1	0.79	2.6	0.93	1.014
57	5f10	0.72	1.9	0.64	1.03
57	1e6-	0.84	1.49	0.984	0.9871
57	1h2	0.94	1.43	0.68	0.991
57	2a6	1.08	1.08	0.89	0.9976

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Experiment      Clone                      Li            tau            Km            S  
                         ID

58	1g6	1.57	8.9	1.78	1.02
<b>58</b>	<b>0a5</b>	<b>1.53</b>	<b>8.5</b>	<b>1.56</b>	<b>1.05</b>
58	1b1	0.84	8.5	0.6	1.04
58	3g1	1	7.34	0.62	1.006
58	0f3	1.31	6.9	0.57	0.98
58	3e12-	1.06	6.3	0.47	0.996
58	0c7	1.9	4	0.64	1.06
58	0d1	1.03	3.76	0.49	1.03
58	3c7	1.49	3.4	0.55	1.04
58	2a2	1.4	2.2	0.5	1.05
58	2a8	3.2	2	0.81	1.05
58	0f2	2.2	1.92	0.45	1.04

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58	1b4	5.1	1.87	1.08	1.09
58	2b3	2.7	1.55	0.57	1.04
58	4g1	4.9	1.2	0.72	1.06

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Control is Luc58-0A5 at 58°C

Experiment	Clone ID	Li	tau	Km	S
61	4e9-	1.03	1.84	0.76	1.01
61	1f1	1.02	1.43	0.7	1
61	2e12-	1.56	1.34	0.48	1.003
61	2f2	1.5	1.3	0.32	1.01
61	6b4	1.2	1.26	0.88	0.98
61	4c10	1.46	1.12	1.06	0.99
61	4g11	1.31	1.03	1.43	1.03
61	2f1	1.41	1.02	0.79	0.995
61	2g1	1.3	1	1.17	1

Experiment	Clone ID	Li	tau	Km	S
65	6g12	0.87	2.3	0.73	0.9605
65	1h6	0.84	2.2	1.62	0.9598
65	7f5	1.2	1.56	2.07	1.0087
65	5g5	2.3	1.49	0.45	0.9985
65	7h2	1.56	1.27	0.91	1.0658
65	7b2	1.98	1.16	0.6	0.9289
65	0g9	1.36	1.09	1.46	0.9927
65	6c7	1.48	1.06	0.86	0.9967
65	1e12-	1.59	1.05	1.03	0.9582
65	4e2-	1.21	1.05	1.11	0.943
65	6a10	1.7	1.04	0.93	0.992
65	4b9	1.48	1.04	1.61	1.0009
65	6c1	1.36	1.02	0.72	0.9978

Experiment	Clone ID	Li	tau	Km	S
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68	2g6	1.39	3.9	1.17	0.9955
68	4g3	2	2.5	0.27	0.9927
68	5a3	1.04	1.64	0.65	0.8984
68	2b7	1.04	1.64	5.2	0.9237
68	5d10	2.75	1.36	0.73	1.0078
68	7d12	1.85	1.32	0.66	1.0084
68	7b9	1.8	1.19	0.56	1.0052
68	7b3	1.2	1.16	0.55	0.9951
68	1g10	1.48	1.05	1.22	1.0025

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Experiment	Clone ID	Li	tau	Km	S
70	2a7	1.94	4.6	0.7	1.0015
70	3d6	3.5	4.2	0.18	1.03
70	4f8	1.87	4.2	0.69	0.9979
70	7h5	2.4	2.6	0.18	1
70	5h6	3.1	2.3	0.6	0.999
70	7d6	3	2.2	2.29	0.9989
70	5a3	3.1	1.5	0.18	1.0058
70	7d2	2.5	1.4	0.66	1.0126
70	3h7	3.2	1.22	0.23	1.002
70	0h5	2.5	1.15	0.36	0.9992
70	0d7	1.86	1	1.83	0.993
70	1g12	2.42	1	0.26	0.965

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Experiment	Clone ID	Li	tau	Km	S
71	1d10	1.6	4.5	1.06	1.0065
71	6f11	1.8	4.3	0.98	0.953
71	7h4	3.4	3.6	0.56	1.0045
71	4h3	3.1	3.1	0.42	1.0171
71	1h5	1.31	3.01	1.31	0.9421
71	5e4-	5.4	2.3	0.35	0.994
71	5c1	2.2	2.3	0.89	0.9746
71	0h7	3.6	1.8	0.59	1.0197

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71	6h9	23.7	1.71	0.91	1.0064
71	7e3-	5.3	1.7	0.7	1.0028
<b>71</b>	<b>5d4</b>	<b>11.1</b>	<b>1.48</b>	<b>0.35</b>	<b>1.0213</b>
71	2e3-	4	1.47	0.45	0.9654
71	6h11	17.7	1.15	2.8	1.0064
71	2e10-	3	1.1	0.66	0.9588
71	2g2	4.4	1.01	0.44	1.0046

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Control is Luc71-5D4 at 60°C

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Experiment	Clone ID	Li	tau	Km	S
72	2g6	0.38	3.1	1.58	1.0052
72	5f12	0.81	1.53	1.02	0.9678
72	0d7	0.76	1.44	1.4	0.9838
72	5c12	0.87	1.43	1.04	0.9718
72	1e1-	1.04	1.41	1.15	0.9956
72	5b12	0.83	1.41	1.02	0.9731
72	0b7	1.11	1.04	0.91	1.0049
72	3b4	0.49	1.03	2.2	0.9581

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Experiment	Clone ID	Li	tau	Km	S
73	2h8	0.85	1.9	1.08	1.0123
73	4e6-	0.95	1.76	0.94	0.9939
73	3g8	0.86	1.53	1.04	1
73	1g3	1.7	1.14	0.97	0.9921

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Experiment	Clone ID	Li	tau	Km	S
74	2a9	0.96	1.77	0.86	0.999
74	4e10-	0.8	1.36	1.33	0.09897
74	0d5	1.69	1.28	0.61	0.9927
74	6g7	1.75	1.07	1.33	1.0022
74	5d8	0.46	1.06	0.95	0.899
74	5e7-	1.22	1.05	0.87	0.9977

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74	6e1-	1.19	1.02	0.96	0.999
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	Experiment	Clone ID	Li	tau	Km	S
5	76	6c3	2.3	6.4	1.2	0.9865
	76	2a9	0.93	4.7	1.08	0.999
	76	3h9	1.26	2.6	1.02	0.9973
	76	0b10	1.52	2.4	1.4	0.992
	76	0h9	1.71	1.44	1.05	1.018
10	76	2e9-	0.44	1.15	1.2	0.9318
	76	0e10-	1.67	1.1	1.02	1.014
	76	0c10	1.13	1.05	1	0.9974
	76	3e8-	1.35	1.03	1.1	0.9894
	76	0d12	0.69	1	0.92	0.932
15	76	0f10	0.62	1	1.2	0.9478

	Experiment	Clone ID	Li	tau	Km	S
20	78	1e1-	0.54	8.9	1.15	0.9877
	78	0h7	1.4	5	0.97	1.014
	78	0a6	1	4.3	1.5	0.9967
	<b>78</b>	<b>0b10</b>	<b>1.93</b>	<b>2</b>	<b>1</b>	<b>0.9926</b>
	78	0f11	1.6	2	0.91	0.9905
	78	3f1	2.4	1.7	1.09	0.9936
25	78	2b4	1.97	1.36	0.98	1.0094
	78	5b3	3.2	1.19	1.03	0.9735
	78	2g12	2.5	1.03	1	1.0134
	78	0h2	1.6	1	1.15	1.0168

30

Control is Luc78-0B10 at 62°C

	Experiment	Clone ID	Li	tau	Km	S
	82	2g12	0.9811	2.09	0.8851	0.9939
	82	4b9	1.0845	1.8419	0.8439	1.0078
35	82	0d1	0.7622	1.5171	1.11	0.9998



5

82	3g1	0.8805	1.504	0.9629	0.9927
82	1d1	0.9741	1.4497	0.8936	0.9986
82	1e8-	0.8206	1.4433	0.9876	0.9968
82	0h9	1.1355	1.3626	0.9171	1.0094
82	2c6	1.0931	1.3402	0.9482	1.0022
82	3g9	1.0364	1.251	0.968	1.0009
82	4h8	0.8816	1.1667	0.9165	1.0045
82	0a10	1.0535	1.1128	1.0413	1
82	4g1	1.4305	1.0862	1.1734	1.0059

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Experiment	Clone ID	Li	tau	Km	S
84(121)	6h7	0.3755	29.3639	2.3636	0.8905
84(121)	2h9	0.4264	28.7958	1.819	0.904
84(121)	3f7	0.4161	25.3058	1.8079	0.8988
84(121)	2h10	0.9667	14.4658	0.8073	0.9947
84(121)	3a2	0.3329	12.6	2.5444	0.855
<b>84(121)</b>	<b>3a6</b>	<b>1.2299</b>	<b>7.2384</b>	<b>0.7866</b>	<b>1.0046</b>
84(121)	5b12	1.0535	6.0315	0.7824	1.0056
84(121)	5a7	1.0413	4.9054	0.8864	1.0071
84(121)	3d2	0.2032	4.8	2.4623	0.7973
84(121)	2a9	1.0847	4.7486	0.7746	1.0051
84(121)	5e11-	1.1918	4.0988	0.872	1.008
84(121)	7h2	0.9115	3.9929	0.909	1.0077
84(121)	3b5	1.2014	3.8251	0.7509	1.0086
84(121)	1f8	1.07	3.06	0.8276	1.0093
84(121)	2e2-	1.4356	1.9315	0.7863	1.0175

30

Control is Luc84-3A6 at 64°C

35

Experiment	Clone ID	Li	tau	Km	S
85(86)	2a2	0.2266	12.9013	3.326	0.8705
<b>85(86)</b>	<b>4f12</b>	<b>1.1167</b>	<b>4.7851</b>	<b>0.7439</b>	<b>1.0092</b>
85(86)	4e9-	1.0869	4.4953	0.8539	1.0068
85(86)	1f11	0.6994	4.0976	0.842	1.0124

29

5	85(86)	5a4	1.2273	4.09	0.9683	1.0098
	85(86)	3e10-	0.8902	3.5342	0.8106	1.0069
	85(86)	3e12-	1.0512	3.4883	0.853	1.0054
	85(86)	5e4-	0.9562	3.3886	1.0328	1.0069
	85(86)	0e6-	0.1494	3.0145	3.6293	0.8269
10	85(86)	6b1	0.7615	2.5712	0.8695	1.0055
	85(86)	6h7	1.0285	2.5401	0.8963	1.0057
	85(86)	4b11	0.9816	2.3899	0.7927	1.0063
	85(86)	6d7	1.1087	2.0607	0.9042	1.0088
	85(86)	2e10-	0.3028	2.0603	1.9649	0.8738
	85(86)	2a9	1.448	1.1819	0.9722	1.0046

Control is Luc85-4F12 at 65°C

15	Experiment	Clone ID	Li	tau	Km	S
	88	3c1	1.4439	2.0938	0.9874	0.9976
	88	6g1	1.0184	1.2665	1.2184	1.0019
	88	3e4-	1.331	1.0996	1.0669	0.9983

20

	Experiment	Clone ID	Li	tau	Km	S
	89	1a4	1.2565	2.4796	1.0338	0.997
	89	3b1	0.7337	1.9976	0.9628	1.0001
	89	2b12	1.0505	1.8496	1.0069	1.0012
25	89	0b5	1.5671	1.1362	1.0912	0.9995
	89	1f1	1.378	1.1018	0.9804	0.996
	89	2f1	1.4637	1.0894	0.9189	0.9992

30

	Experiment	Clone ID	Li	tau	Km	S
	90	0f1	1.4081	1.3632	1.027	0.9987
	90	1b5	1.4743	1.1154	1.0812	1.0011
	90	6g5	1.2756	1.0605	1.0462	1.0012
	90	5e6-	1.0556	1.0569	1.1037	1.0011
35	90	4e3-	1.2934	1.0291	1.0733	1.0002

To evaluate the impact of directed evolution on enzyme function, clones from the beginning, middle and end of the process (Table 2) were purified and analyzed. The clones selected for this analysis were Luc[T249M], Luc49-7C6, and Luc78-0B10. Another clone, Luc90-1B5, created by a subsequent strategy of oligonucleotide-directed mutagenesis and screening was also purified for analysis.

**Table 2:** Thermostability Of Luciferase Activity At Different Temperatures (Half-Life In Hours)

10

	<i>Room Temperature*</i>	<i>37°C</i>	<i>50°C</i>	<i>60°C</i>
Luc[T249M]	110	0.59	0.01	
Luc49-7C6	430	68	31	6.3
Luc78-0B10	3000	220	47	15

15 \* about 25°C

The effect of directed evolution on thermostability was dramatic. At high temperatures, where the parent clone was inactivated almost instantaneously, the mutant enzymes from the related clones showed thermostability over several hours (see also Table 1, and Figures 1-3, 5-8, 11, 13, 50-52 and 60). Even at room temperature, these mutants are several fold more thermostable than the parent enzyme (see also Figures 4, 9-10, 12, 53, and 62). Subsequent analysis of Luc90-1B5 showed this enzyme to be even more thermostable, having a half-life of 27 hours at 65°C when tested under the same buffer conditions (Figure 16A). With some optimization of buffer conditions, this enzyme showed very little activity loss at 65°C over several hours (citrate buffer at pH 6.5; Figure 16A). This luciferase was stable at 22°C over several weeks when incubated at pH 6.5 (Figure 16B). At times over 100 days at 4°C, the mutant enzymes had increased thermostability. At times of less than 15 days at 4°C, the thermostabilities of the mutants Luc49-7C6 and Luc78-0B10 were not distinguishable from the parent enzyme (Figure 49).

Kajiyama and Nakamo (1993) showed that a single amino acid substitution of A at position 217; to either I, L, or V, in the firefly luciferase from *Luciola*

*lateralis*, resulted in a luciferase having increased thermostability. Substitution with leucine produced a luciferase that maintained 70% of its activity after incubation for 1 hour at 50°C. All of the enzymes of the present invention created through directed evolution, are much more stable than this *L. lateralis* mutant. One clone, Luc90-1B5, maintains 75% activity after 120 hours (5 days) incubation under similar conditions (50°C, 25 mol/L citrate pH 6.5, 150 mmol/L NaCl, 1 mg/mL BSA, 0.1 mmol/L EDTA, 5% glycerol). Interestingly, the *LucPpe2* reported by Leach et al. already contains isoleucine at the homologous position described for the *L. lateralis* mutant.

Although thermostability was the characteristic of interest, clones were selected based on the other enzymological parameters in the screens. By selecting clones having greater luminescence expression, mutants were found that yielded greater luminescence intensity in colonies of *E. coli*. However, the process showed little ability to alter the kinetic profile of luminescence by the enzymes. This failure suggests that the ability to support steady-state luminescence is integral to the catalytic mechanism, and is not readily influenced by a cumulative effect of many amino acids.

Substrate binding was screened by measuring an apparent composite  $K_m$  (see Example 2) for luciferin and ATP. Although the apparent composite  $K_m$  remained relatively constant, later analysis showed that the individual  $K_m$ 's systematically changed. The  $K_m$  for luciferin rose while the  $K_m$  for ATP declined (Table 3). The reason for this change is unknown, although it can be speculated that more efficient release of oxyluciferin or luciferin inhibitors could lead to more rapid enzyme turnover.

Each point mutation, on its own, increases (to a greater or lesser extent) the thermostability of the mutant enzyme relative to the wild-type luciferase. The cumulative effect of combining individual point mutations yields mutant luciferases whose thermostability is greatly increased from the wild-type, often on the order of a magnitude or more.

**Table 3:** Michaelis-Menten Constants for Mutants Created by Directed Evolution

		$K_m$ -luciferin	$K_m$ -ATP
5	Luc[T249M]	0.32 $\mu$ M	18 $\mu$ M
	Luc49-7C6	0.99 $\mu$ M	14 $\mu$ M
	Luc78-0B10	1.6 $\mu$ M	3.4 $\mu$ M
	Luc90-1B5	2.2 $\mu$ M	3.0 $\mu$ M

The following examples illustrate the methods and compositions  
 10 of the present invention and their embodiments.

### **EXAMPLE 1**

#### **Producing Thermostable Luciferases Of The Present Invention**

##### Mutagenesis Method.

15 An illustrative mutagenesis strategy is as follows: From the "best" wild-type luciferase clone, that is a clone with increased thermostability and not appreciably diminished values for other parameters, random mutagenesis was performed by three variations of error-prone PCR. From each cycle of random mutagenesis, 18 of the best clones were selected. DNA was prepared from these  
 20 clones yielding a total of 54 clones. These clones represent new genetic diversity.

These 54 clones were combined and recombination mutagenesis was performed. The 18 best clones from this population were selected.

25 These 18 clones were combined with the 18 clones of the previous population and recombination mutagenesis was performed. From this screening, a new luciferase population of 18 clones was selected representing 6 groups of functional properties.

30 In this screening the new mutations of the selected 54 clones, either in their original sequence configurations or in recombinants thereof, were screened a second time. Each mutation was analyzed on the average about 10 times. Of the 90 clones used in the recombination mutagenesis, it was likely that at least 10 were functionally equivalent to the best clone. Thus, the best clone or recombinants thereof should be screened at least 100 times. Since this was greater than the number of clones used in the recombination, there was

significant likelihood of finding productive recombination of the best clone with other clones.

#### Robotic Processing Methods.

Heat transfers were controlled in the robot process by using thick  
5 aluminum at many positions where the 96-well plates were placed by the robotic arm. For example, all shelves in the incubators or refrigerator were constructed from ¼ inch aluminum. One position in particular, located at room temperature, was constructed from a block of aluminum of dimensions 4.5 x 7 x 6.5 inches. When any 96-well plate was moved from a high temperature (e.g., incubators) or  
10 low temperature (e.g., refrigerator) to a device at room temperature, it was first placed on the large aluminum block for temperature equilibration. By this means, the entire plate would rapidly reach the new temperature, thus minimizing unequal evaporation for the various wells in the plate due to temperature differences. Heat transfers in a stack of 96-well plates placed in an  
15 incubator (e.g., for overnight growth of *E. coli*) were controlled by placing 1 mm thick sheets of aluminum between the plates. This allowed for more efficient heat transfer from the edges of the stack to the center. Mixing in the robotic process was controlled by having the plate placed on a shaker for several second after each reagent addition.

20 Please refer to Figure 14 for a schematic of the order in which the plates are analyzed and to Figure 15 for a robotic apparatus which can be programmed to perform the following functions:

1. Culture Dilution Method.

25 A plate with lid (Falcon 3075) containing cells (*E. coli* JM109) is placed on a shaker and mixed for 3-5 minutes.

A plate (with lid) is obtained from a carousel and placed in the reagent dispenser. 180 µl of media (M9 minimal media) is added after removing the lid and placing on the locator near the pipetter. The plate is then placed in the pipetter.

30 The plate on the shaker is placed in the pipetter, and the lid removed and placed on the locator. Cells are transferred to the new plate using pipetting procedure (see "Dilution of Cells into New Cell Plate").

The lids are replaced onto both plates. The new plate is placed in the refrigerator and the old plate is returned to the carousel.

2. Luminescence Assay Method.

5 A plate containing cells is retrieved from the carousel and placed on the shaker for 3-5 minutes to fully mix the cells. The cells tend to settle from solution upon standing.

To measure Optical Density (O.D.), the plate is moved from the shaker to the locator near the luminometer; the lid is removed and the plate placed into the luminometer. The O.D. is measured using a 620 nm filter.

10 When it is finished, the plate is then placed in the refrigerator for storage.

The above steps are completed for all plates before proceeding with subsequent processing.

To prepare a cell lysate, the plate of cells is first retrieved from the refrigerator and mixed on the shaker to resuspend the cells. A new plate from  
15 the carousel without a lid is placed in the reagent dispenser and 20  $\mu$ l of Buffer A is added to each well. This is placed in the pipetting station.

The plate of cells in the shaker is placed in the pipetting station. A daughter plate is prepared using pipetting procedure (see "Pipetting Cells into the Lysis Plate") to prepare a daughter plate of cells.

20 After pipetting, the new daughter plate is placed on the shaker for mixing.

After mixing, the Lysate Plate is placed into a solid CO<sub>2</sub> freezing station to freeze the samples. The plate is then moved to the thaw block to thaw for 10 minutes.

25 The plate is then moved to the reagent dispenser to add 175  $\mu$ l of Buffer B, and then mixed on the shaker for about 15 minutes or more. The combination of the freeze/thaw and Buffer B will cause the cells to lyse.

A new plate with a lid from the carousel is used to prepare the dilution plate from which all assays will be derived. The plate is placed in the reagent  
30 dispenser and the lid removed to the locator near the pipetter. 285  $\mu$ l of Buffer C is added to each well with the reagent dispenser, then the plate is placed in the pipetting station.

The Lysate Plate in the shaker is moved to the pipetting station and pipetting procedure (see "Dilution from Lysis Plate to Incubation Plate") is used. After pipetting, the new daughter plate is placed on the shaker for mixing. The Lysate Plate is discarded.

- 5 Two white assay plates (Labsystems #9502887) are obtained from the plate feeder and placed in the pipetter. The incubation plate from the shaker is placed in the pipetter, and the lid removed and placed on the nearby locator. Two daughter plates are made using the pipetting procedure (see "Create Pair of Daughter Plates from Incubation Plate"). Afterwards, the lid is replaced on the
- 10 parent plate, and the plate is placed in a high temperature incubator. [ranging from 31°C to about 65°C depending on the clone.]

- One daughter plate is placed in the luminometer and the 1X assay method is used. After the assay, the plate is placed in the ambient incubator, and the second daughter plate is placed in the luminometer. For the second plate, the
- 15 0.02X assay method is used. This plate is discarded, and the first plate is returned from the incubator to the luminometer. The repeat assay method is used (i.e., no reagent is injected). Afterwards, the plate is again returned to the ambient incubator.

- The above steps are completed for all plates before proceeding with
- 20 processing.

To begin the second set of measurements, the plate from the high temperature incubator is placed in the shaker to mix.

- The plate in the ambient incubator is returned to the luminometer and the repeat assay method is again used. The plate is returned afterwards to the
- 25 ambient incubator.

- Two white assay plates again are obtained from the plate feeder and placed in the pipetter. The plate on the shaker is placed in the pipetter, and the lid removed and placed on the nearby locator. Two daughter plates are again made using the pipetting procedure (see "Create Pair of Daughter Plates from
- 30 Incubation Plate"). Afterwards, the lid is replaced on the parent plate, and the plate is returned to the high temperature incubator.

One daughter plate is placed in the luminometer and the 1X assay method is again used. The plate is discarded after the assay. The second daughter plate is



then placed in the luminometer and the 0.06X assay method is used. This plate is also discarded.

The above steps are completed for all plates before proceeding with processing.

- 5            In the final set of measurements, the plate from the high temperature incubator is again placed in the shaker to mix.

The plate in the ambient incubator is returned to the luminometer and the repeat assay method is again used. The plate is discarded afterwards.

- 10           One white assay plate is taken from the plate feeder and placed in the pipetter. The plate from the shaker is placed in the pipetter, and the lid removed and placed on the nearby locator. One daughter plate is made using the pipetting procedure (see "Create Single Daughter Plate from Incubation Plate"). The lid is replaced on the parent plate and the plate is discarded.

- 15           The daughter plate is placed in the luminometer and the 1X assay method is used. The plate is discarded after the assay.

#### Buffers and Assay Reagents

Buffer A: 325 mM  $K_2HPO_4$ ; 6.5 mM CDTA; 0.1% Triton X-100

- 20           Buffer B: 1X CCLR (Promega E153A); 1.25 mg/ml lysozyme; 0.04% gelatin

Buffer C: 10 mM HEPES; 150 mM NaCl; 1 mg/ml BSA; 5% glycerol; 0.1 mM EDTA

1X Assay reagent: 5  $\mu$ M Luciferin; 175  $\mu$ M ATP; 20 mM Tricine, pH 8.0; 0.1 mM EDTA

- 25           0.02X Assay reagent: 1:50 dilution of 1X Assay reagent

0.06X Assay reagent: 1:16.7 dilution of 1X Assay reagent

#### Pipetting Procedures

##### A. Pipetting Cells Into the Lysis Plate

- 30           Non-aseptic procedure using fixed tips

On the pipetter deck:

-place a plate containing approximately 200  $\mu$ l JM109 cells per well without lid

-Lysate Plate containing 20 µl of Buffer A

Procedure:

1. Move the tips to the washing station and wash with 1 ml.
2. Move to the cell plate and withdraw 60 µl.
- 5 3. Move to the Lysate Plate and dispense 45 µl.
4. Repeat steps 1-3 for all 96 samples.
5. At the conclusion of the procedure, step 1 is repeated to clean the tips.

Post-procedure:

- Place Lysate Plate onto the shaker.
- 10 - Place lid on plate with cells and place on carousel.
- Place Lysate Plate into the CO<sub>2</sub> freezer.

B. Dilution From Lysis Plate to Incubation Plate

On the pipetter deck:

- 15 - Lysate Plate containing 240 µl of lysate
- Incubation Plate without lid containing 285 µl of Buffer C

Procedure:

1. Move the tips to the washing station and wash with 0.5 ml.
2. Move to the Lysate Plate and withdraw 30 µl.
- 20 3. Move to the Incubation Plate and dispense 15 µl by direct contact with the buffer solution.
4. Repeat steps 1-3 for all 96 samples.
5. At the conclusion of the procedure, step 1 is repeated to clean the tips.

Post-procedure:

- 25 - Place Incubation Plate on shaker.
- Discard Lysate Plate.

C. Create Pair of Daughter Plates From Incubation Plate

This procedure is done twice

- 30 On the pipetter deck:

- Incubation Plate containing 100-300 µl of solution without lid
- Two empty Assay Plates (white)

Procedure:

1. Move the tips to the washing station and wash with 0.5 ml.
2. Move to the Incubation Plate and withdraw 50  $\mu$ l.
3. Move to the first Assay Plate and dispense 20  $\mu$ l.
- 5     4. Move to the second Assay Plate and dispense 20  $\mu$ l.
5. Repeat steps 1-4 for all 96 samples.
6. At the conclusion of the procedure, step 1 is repeated to clean the tips.

Post-procedure:

1. Replace lid on Incubation Plate.
- 10     2. Place Incubation Plate in incubator.
3. Place first Assay Plate in luminometer.
4. Place second Assay Plate on carousel.

D. Create Single Daughter Plate From Incubation Plate15     On the pipetter deck:

- Place incubation Plate containing 100-300  $\mu$ l of solution without lid and
- Empty Assay Plate (white)

Procedure:

1. Move the tips to the washing station and wash with 0.5 ml.
- 20     2. Move to the Incubation Plate and withdraw 40  $\mu$ l.
3. Move to the Assay Plate and dispense 20  $\mu$ l.
4. Repeat steps 1-3 for all 96 samples.
5. At the conclusion of the procedure, step 1 is repeated to clean the tips.

Post-procedure:

- 25     - Discard Incubation Plate and lid on Incubation Plate.
- Place Assay Plate in luminometer.

E. Dilution of Cells Into New Cell Plate

Aseptic procedure using fixed tips

30     On the pipetter deck:

- plate containing approximately 200  $\mu$ l of cells without lid
- new cell plate containing 180  $\mu$ l of Growth Medium without lid

Procedure:

1. Move to the cell plate and withdraw 45  $\mu$ l.
2. Move to the Cell Plate and dispense 20  $\mu$ l volume by direct liquid-to-liquid transfer.
- 5 3. Move to waste reservoir and expel excess cells.
4. Move to isopropanol wash station aspirate isopropanol to sterilize tips.
5. Move to wash station, expel isopropanol and wash tips.
6. Repeat steps 1-4 for all 96 samples.

Post-procedure:

- 10 - Replace lid on original plate of cells and place onto carousel.
- Replace lid on new cell plate and place into refrigerator.

Notes:

- This procedure is used to prepare the cell plates used in the main analysis procedure. 180  $\mu$ l of M9 minimal growth medium is added by the reagent
- 15 dispenser to each of the new cell plates just prior to initiating the pipetting procedure. The dispenser is flushed with 75% isopropanol before priming with medium. The medium also contains selective antibiotics to reduce potential contamination.

20 Luminometer ProceduresA. 1X Assay Method

1. Place plate into luminometer.
2. Inject 100  $\mu$ l of 1X Assay reagent.
3. Measure luminescence for 1 to 3 seconds.
- 25 4. Repeat for next well.
5. Continue until all wells are measured.

B. 0.02X Assay Method

1. Place plate into luminometer.
- 30 2. Inject 100  $\mu$ l of 0.02X Assay reagent.
3. Measure luminescence for 1 to 3 seconds.
4. Repeat for next well.
5. Continue until all wells are measured.

C. 0.06X Assay Method

1. Place plate into luminometer.
2. Inject 100  $\mu$ l of 0.06X Assay reagent.
3. Measure luminescence for 1 to 3 seconds.
- 5 4. Repeat for next well.
5. Continue until all wells are measured.

D. Repeat Assay

1. Place plate into luminometer.
- 10 2. Measure luminescence for 1 to 3 seconds.
3. Repeat for next well.
4. Continue until all wells are measured.

In Vivo Selection Method

- 15 Five to seven nitrocellulose disks having 200-500 colonies per disk (1000-3500 colonies total) are screened per 2 microplates (176 clones) (Wood and DeLuca, 1987). The clones are screened at high temperatures using standard screening conditions.

- Eight positions in each microplate are reserved from a reference clone using the "best" luciferase (the parent clone for random mutagenesis and codon mutagenesis). The positions of the reserved wells is shown as "X" below.

25

```

XoooooooooX
oooooooooooo
oooXooooXooo
oooooooooooo
oooooooooooo
oooXooooXooo
oooooooooooo
XoooooooooX
30

```

The reference clones are made by placing colonies from DNA transformed from the parent clone into the reference wells. To identify these

wells prior to inoculation of the microplate, the wells are marked with a black marking pen on the bottom of each well.

#### Screening Selection Criteria

The following criteria were used for screening purposes. The

- 5 temperature chosen for the enzyme stability parameter was such that the parent enzyme would decay 100 to 1000 fold over 10 hours (see Table 1). Criteria 1 is achieved manually; data for criteria 2-6 is generated by robotic analysis. For all criteria, the maximum value as described is selected.
- 10 1. ***In vivo* screen.** The brightest clones are selected at a given temperature.
  2. **Expression/specific activity.** The value for normalized luminescence is calculated as the ratio of luminescence to optical density. The value is reported as the ratio with the reference value.
  - 15 3. **Enzyme stability.** Measurements of normalized luminescence of the incubated samples (3 taken over about 15 hours) are fitted to  $\ln(L) = \ln(L_0) - (t/\tau)$ , where L is normalized luminescence and t is time.  $\tau$  is a measure of the enzyme stability. The value is reported as the ratio with the reference value, and the correlation
  - 20 coefficients are calculated.
  4. **Substrate binding.** Measurements of normalized luminescence with 1X and 0.02X are taken at the initial reading set, and 1X and 0.06X are taken at the 5 hour set. The ratio of the 0.02X:1X and 0.06X:1X gives the relative luminescence at 0.02X and 0.06X
  - 25 concentrations. These values, along with the relative luminescence at 1X (i.e., 1), are fitted to a Lineweaver-Burk plot to yield the  $K_m:app,total$  for the substrates ATP, luciferin, and CoA. The values are reported as the inverse ratio with the reference value, and the correlation coefficients are calculated.
  - 30 5. **Signal stability.** The luminescence of the initial 1X luminescent reactions are re-measured 3 additional times over about 15 hours. These values are fitted to  $\ln(L) = \ln(L_0) - (t/\tau)$  and the integral over

t (15 hours) is calculated. Signal stability is then calculated as  $S = (1 - \ln(L)/L_0 t)^2$ . The values are reported as the inverse ratio with the reference value, and the correlation coefficients are calculated.

- 5            6. **Composite fitness.** The values of criteria 2 through 5 are combined into a single composite value of fitness (or commercial utility). This value is based on a judgment of the relative importance of the other criteria. This judgment is given below:

10	<u>Criteria</u>	<u>Relative Value</u>
	Enzyme Stability	5
	Signal Stability	2
	Substrate Binding	2
	Expression/Activity	1

15

The composite,  $C = \text{Sum}(\text{criteria 2-5 weighted by relative value, e.g., more weight is on stability because that was a major goal})$ .

## **EXAMPLE 2**

20

### **Software**

#### Organize data into SQL database

Each file created by a luminometer (96 well, Anthos, Austria) represents the data from one microplate. These files are stored in the computer controlling the luminometer, and connected to the database computer by a network link.

- 25 From each microplate of samples, nine microplates are read by the luminometer (the original microplate for optical density and eight daughter microplates for luminescence).

Ninety files are created in total; each containing data sets for 96 samples. Each data set contains the sample number, time of each measurement relative to the first measurement of the plate, luminometer reading, and background corrected luminometer reading. Other file header information is also given. The time that each microplate is read is also needed for analysis. This can be obtained from the robot log or the file creation time. A naming convention for

30

the files is used by the robot during file creation that can be recognized by SQL (e.g. YYMMDDPR.DAT where YY is the year, MM is the month, DD is the day, P is the initial plate [0-9], and R is the reading [0-8]).

## 5 Data Reduction and Organization

Normalize luminescence data: For each measurement of luminescence in the eight daughter plates, the normalized luminescence is calculated by dividing the relative light units by the optical density of the original plate. If any value of normalized luminescence is less than zero, assign the value of 0.1 sL where sL is the standard deviation for measurements of normalized luminescence.

Calculate relative measurement time: For each normalized luminescence measurement, the time of the measurement is calculated relative to the first measurement of the sample. For example, the times of all luminescence measurements of sample B6 in plate 7 (i.e., 7:B06) are calculated relative to the first reading of 7:B06. This time calculation involves both the time when the plate is read and the relative time of when the sample is read in the plate.

Calculate enzyme stability ( $\tau$ ): For each sample, use linear regression to fit  $\ln(L_{1x}) = \ln(L_0) - (t/\tau)$  using the three luminescence measurements with 1X substrate concentrations (Plates 1, 5, 8). Also calculate the regression coefficient.

Calculate substrate binding ( $K_{m:app,total}$ ): Using microplates from the first set of readings (Plates 1 and 2), calculate the  $L_{0.02x,rel}$  by dividing measurements made with substrate concentrations of 0.02X by those of 1X. Similarly, calculate the  $L_{0.06x,rel}$  using microplates of the second set of readings (Plates 5 and 6), by dividing measurements made with substrate concentrations of 0.06X by those of 1X.

For each sample, use linear regression to fit  $1/L = (K_{m:app,total}/L_{max:app}) + (1/[S])$  using

	L	[S]
30	$L_{0.02x,rel}$	0.02
	$L_{0.06x,rel}$	0.06
	1 ( $L_{1x,rel}$ )	1



$K_{m:app,total}$  is calculated as the slope/intercept. Also calculate the regression coefficient.

Calculate signal stability (S): For each sample, use linear regression to fit  $\ln(L) = \ln(L_0) - (t/\tau)$  using the four luminescence measurements of the initial microplate with 1X substrate concentrations (Plates 1, 3, 4, and 7). Also calculate the regression coefficient. From the calculated values of  $\tau$  and  $L_0$ , calculate the integral of luminescence by  $\text{int}(L) = \tau L_0 (1 - \exp(-t_f/\tau))$ , where  $t_f$  is the average time of the last measurement (e.g., 15 hours). The signal stability is calculated as  $S = (1 - \text{int}(L)/L_i t_f)^2$ , where  $L_i$  is the initial measurement of normalized luminescence with 1x substrate concentration (Plate 1).

[Note: To correct for evaporation, an equation  $S = (1 + K - \text{int}(L)/L_i t_f)^2$ , may be used where  $1/K = 2(\text{relative change of liquid volume at } t_f)$ .]

Calculate the reference value surfaces: A three dimensional coordinate system can be defined by using the grid positions of the samples within a microplate as the horizontal coordinates, and the calculated values for the samples ( $L_i$ ,  $\tau$ ,  $K_{m:app,total}$ , or  $S$ ) as the vertical coordinates. This three dimensional system is referred to as a "plate map". A smooth surface in the plate maps representing a reference level can be determined by least squares fit of the values determined for the 8 reference clones in each microplate. For each of the 10 initial microplates of samples, respective reference surfaces are determined for the criteria parameters  $L_i$ ,  $\tau$ ,  $K_{m:app,total}$ , and  $S$  (40 surfaces total).

In the least squares fit, the vertical coordinates (i.e., the criteria parameters) are the dependent variables, the horizontal coordinates are the independent variables. A first order surface (i.e.,  $z = ax + by + c$ ) is fitted to the values of the reference clones. After the surface is calculated, the residuals to each reference clone are calculated. If any of these residuals is outside of a given cutoff range, the reference surface is recalculated with omission of the aberrant reference clone.

If a first order surface does not sufficiently represent the values of the reference clones, a restricted second order surface is used (i.e.,  $z = a(x^2 + ky^2) + bx + cy + d$ , where  $k$  is a constant).

Calculate the reference-normalized values: For the criteria parameter of each sample, a reference-normalized value is determined by calculating the ratio

or inverse ratio with the respective reference value. The reference-normalized values are  $L_i / L_{ir}$ ,  $\tau / \tau_r$ ,  $K_{mr} / K_{m:app,total}$ , and  $S_r / S$ , where reference values are calculated from the equations of the appropriate reference surface.

Calculate the composite scores: For each sample, calculate

$$5 \quad C = 5(\tau / \tau_r) + 2(S_r / S) + 2(K_{mr} / K_{m:app,total}) + (L_i / L_{ir}).$$

Determine subgroupings: For the criteria parameters  $L_i$ ,  $\tau$ ,  $K_{m:app,total}$ ,  $S$ , and  $C$ , delimiting values (i.e., bin sizes) for subgroupings are defined as  $gL$ ,  $g\tau$ ,  $gK_m$ ,  $gS$ , and  $gC$ . Starting with the highest values for  $L_i$ ,  $\tau$ , or  $C$ , or the lowest values of  $K_{m:app,total}$  or  $S$ , the samples are assigned to bins for each criteria

10 parameter (the first bin being #1, and so on).

Display sorted table of reference-normalized values: Present a table of data for each sample showing in each row the following data:

- sample identification number (e.g., 7:B06)
- composite score (C)
- 15 - reference-normalized enzyme stability ( $\tau / \tau_r$ )
- correlation coefficient for enzyme stability
- bin number for enzyme stability
- reference-normalized signal stability ( $S_r / S$ )
- correlation coefficient for signal stability
- 20 - bin number for signal stability
- reference-normalized substrate binding ( $K_{mr} / K_{m:app,total}$ )
- correlation coefficient for substrate binding
- bin number for substrate binding
- reference-normalized expression/specific activity ( $L_i / L_{ir}$ )
- 25 - bin number for expression/specific activity

The table is sorted by the composite score (C).

Present sorted table of criteria parameters.

Present a table of data for each sample showing in each row the following data:

- 30 - sample identification number
- composite score (C)
- enzyme stability ( $\tau$ )
- correlation coefficient for enzyme stability

- bin number for enzyme stability
- signal stability (S)
- correlation coefficient for signal stability
- bin number for signal stability
- 5     - substrate binding ( $K_{m:app,total}$ )
- correlation coefficient for substrate binding
- bin number for substrate binding
- expression/specific activity ( $L_i$ )
- bin number for expression/specific activity

10       The table is sorted by the composite score (C); the reference clones are excluded from the table. Same entry coding by standard deviation as described above.

Present sorted table of reference-normalized values

This is the same procedure as the final step of the data reduction  
15    procedure. The table will show:

- sample identification number
- composite score (C)
- reference-normalized enzyme stability ( $\tau/\tau_r$ )
- correlation coefficient for enzyme stability
- 20    - bin number for enzyme stability
- reference-normalized signal stability ( $S_r/S$ )
- correlation coefficient for signal stability
- bin number for signal stability
- reference-normalized substrate binding ( $K_{mr}/K_{m:app,total}$ )
- 25    - correlation coefficient for substrate binding
- bin number for substrate binding
- reference-normalized expression/specific activity ( $L_i/L_{ir}$ )
- bin number for expression/specific activity

The table is sorted by the composite score (C); the reference clones are  
30    excluded from the table. Same entry coding by standard deviation as described above.

Present sorted table of criteria parameters for reference clones

This is the same procedure as described above for criteria parameters, except for only the reference clones. The table will show:

- sample identification number
- 5      - composite score (C)
- enzyme stability ( $\tau$ )
- correlation coefficient for enzyme stability
- bin number for enzyme stability
- signal stability (S)
- 10     - correlation coefficient for signal stability
- bin number for signal stability
- substrate binding ( $K_{m:app,total}$ )
- correlation coefficient for substrate binding
- bin number for substrate binding
- 15     - expression/specific activity ( $L_i$ )
- bin number for expression/specific activity

The table is sorted by the composite score (C). Same entry coding by standard deviation as described above.

Present sorted table of reference-normalized values

- 20      This is the same procedure as described above for reference-normalized values, except for only the reference clones. The table will show:

- sample identification number
- composite score (C)
- reference-normalized enzyme stability ( $\tau/\tau_r$ )
- 25     - correlation coefficient for enzyme stability
- bin number for enzyme stability
- reference-normalized signal stability ( $S_i/S_r$ )
- correlation coefficient for signal stability
- bin number for signal stability
- 30     - reference-normalized substrate binding ( $K_{mr}/K_{m:app,total}$ )
- correlation coefficient for substrate binding
- bin number for substrate binding
- reference-normalized expression/specific activity ( $L_i/L_{ir}$ )

- bin number for expression/specific activity

The table is sorted by the composite score (C). Same entry coding by standard deviation as described above.

#### Sort table

- 5 Any table may be sorted by any entries as primary and secondary key.

#### Display histogram of table

For any table, a histogram of criteria parameter vs. bin number may be displayed for any criteria parameter.

#### Display plate map

- 10 For any plate, a plate map may be displayed showing a choice of:

- any luminescence or optical density measurement

- $L_i$

- $L_i$  reference surface

- $L_i / L_{ir}$

- 15 -  $\tau$

- $\tau$  reference surface

- $\tau / \tau_r$

- correlation coefficient of  $\tau$

- S

- 20 - S reference surface

- $S_r / S$

- correlation coefficient of S

- $K_{m:app,total}$

- $K_m$  reference surface

- 25 -  $K_{mr} / K_{m:app,total}$

- correlation coefficient for  $K_{m:app,total}$

- composite score (C)

The plate maps are displayed as a three dimensional bar chart. Preferably, the bars representing the reference clones are indicated by color or some other

- 30 means.

#### Display drill-down summary of each entry

For  $L_i$ ,  $\tau$ ,  $K_{m:app,total}$ , and S, any entry value in a table may be selected to display the luminescence and optical density reading underlying the value

calculation, and a graphical representation of the curve fit where appropriate. Preferably the equations involved and the final result and correlation coefficient will also be displayed.

5  $L_i$  or  $L_i/L_r$ . Display the optical density and luminescence value from the chosen sample in Plate 0 and Plate 1.

$\tau$  or  $\tau/\tau_r$ . Display the optical density and luminescence value from the chosen sample in Plate 0, Plate 1, Plate 5, and Plate 8. Display graph of  $\ln(L/X)$  vs.  $t$ , showing data points and best line.

10  $S$  or  $S_r/S$ . Display the optical density and luminescence value from the chosen sample in Plate 0, Plate 1, Plate 3, Plate 4, and Plate 7. Display graph of  $\ln(L)$  vs.  $t$ , showing data points and best line.

$K_{m:app,total}$  or  $K_{mr}/K_{m:app,total}$ . Display the optical density and luminescence value from the chosen sample in Plate 0, Plate 1, Plate 2, Plate 5, and Plate 6. Display graph of  $1/L$  vs.  $1/[S]$ , showing data points and best line.

15

### EXAMPLE 3

#### Preparation Of Novel Luciferases

The gene shown in Figure 45 contains a single base pair mutation which encodes an amino acid substitution at position 249, T to M. This clone has a spectral maximum of 552 nm which is yellow shifted from the sequence of Luc.

20 This mutant was selected as an original template because it produces about 5 times brighter luminosity *in vivo* which allowed for more efficient screening.

#### C-terminus mutagenesis

To eliminate the peroxisome targeting signal (SKL), the L was mutated to a STOP codon and the 3 codons immediately upstream were randomized  
25 according to the oligonucleotide mutagenesis procedure described herein. The mutagenic oligonucleotide designed to accomplish this also introduces a unique SpeI site to allow mutant identification without sequencing. The mutants were screened *in vivo* and 13 colonies picked, 12 of which contained the SpeI site.

#### N-terminus mutagenesis

30 To test if expression could be improved, the 3 codons immediately downstream from the initiation Met were randomized as described herein. The mutagenic oligo designed to accomplish this also introduces a unique ApaI site

to allow mutant identification without sequencing. Seven clones were selected, and six of the isolated plasmids were confirmed to be mutants.

#### Shuffling of C- and N-terminus mutants

The C- and N-terminus mutagenesis were performed side-by-side. To  
5 combine the N- and C-terminus mutations, selected clones from each  
mutagenesis experiment were combined with the use of recombination  
mutagenesis according to the recombination mutagenesis protocol described  
herein. The shuffled mutants were subcloned into amp<sup>S</sup> pRAM backbone and  
screened in DH5 F'IQ (BRL; Hanahan, 1985). A total of 24 clones were picked,  
10 only 4 contained both the N- and C- terminus mutations. These 4 clones were  
used as templates for randomization of the cysteine positions in the gene.

#### Mutagenesis to randomize cysteine positions/Random mutagenesis and recombination mutagenesis in the Luc gene

There are 7 cysteine positions in LucPpe2. It is known that these  
15 positions are susceptible to oxidation which could cause destabilization of the  
protein. Seven oligonucleotides were ordered to randomize the cysteine  
positions.

The oligonucleotides were organized into two groups based upon the  
conservation of cysteine in other luciferase genes from different families. Group  
20 1 randomizes the conserved cysteine positions C-60, C-80, and C-162. Group 2  
randomizes cysteines that are not strictly conserved at positions C-38, C-127, C-  
221, and C-257.

The four selected templates from the N- and C-terminus mutagenesis  
were sub-cloned into an ampicillin-sensitive backbone and single-stranded DNA  
25 was prepared for each of the templates. These templates were combined in equal  
amounts and oligonucleotide mutagenesis was completed as described herein. It  
was determined by plating an aliquot of the mutS transformation prior to  
overnight incubation that each of the 2 groups contained  $2 \times 10^4$  independent  
transformants. MutS-DNA was prepared for the 2 groups and was then  
30 transformed into JM109 cells for screening. Mutants from group 1 were  
screened *in vivo* and picks were made for a full robotic run. Five clones were  
selected that had improved characteristics. Mutants from group 2 were screened  
*in vivo* and picks were made for a full robotic run. The temperature incubator on

the robot was set at 33°C for this set of experiments. Ten clones were selected that had improved characteristics. The fifteen best picks from both groups of the cysteine mutagenesis experiments were shuffled together as described herein and 18 of the best clones were selected after robotic processing.

5       The “best” clone from the above experiment (Luc31-1G8) was selected as a template for subsequent rounds of mutagenesis. (The high temperature robot incubator temperature was set to 42°C.) Another complete round of mutagenesis was completed.

10       The 18 best clones from the above mutagenesis were picked and clone (Luc39-5B10) was selected as the best clone and was used as a template for another round of mutagenesis. (The high temperature robot incubator temperature was set at 49°C).

15       After this cycle, 6 of the best clones were selected for sequencing (the nucleotide sequence and inferred amino acid sequence of five of the clones is shown in Figures 22-26 and 27-31, respectively). Based upon the sequence data, nine positions were selected for randomization and seven oligos were designed to cover these positions. Based upon data generated from the robot, it was determined that the best clone from the group of six clones that were sequenced was clone (Luc49-7C6, Figures 22 and 27). The luciferase gene from this clone  
20       was sub-cloned into an ampicillin-sensitive pRAM backbone and single stranded DNA was prepared. The randomization of the selected positions was completed according to the oligonucleotide mutagenesis procedure listed herein.

25       The randomization oligonucleotides were divided into 4 groups, and transformants from these experiments were picked and two robotic runs were completed. Ten clones were selected from the two experiments. (The high temperature robot incubator temperature on robot was set at 56°C).

      The best 10 picks from the above two experiments, and the best 18 picks from the previous population of clones were shuffled together (recombination mutagenesis protocol).

30       The 18 best clones were selected and clone Luc58-0A5 was determined to be the best clone. This clone was then used as a template for another round of mutagenesis. The high temperature robot incubator temperature was set at 58°C.



Clone Luc71-504 was selected as a new lead clone and another round of mutagenesis was completed. Incubator set at 60°C.

The best 18 picks were selected. The nucleotide sequence and inferred amino acid sequence of 4 clones from experiment 78 are shown in Figures 32-35 and 36-39, respectively, and the best clone from this group was determined to be clone Luc78-0B10. The thermostability of clones at various temperatures is presented in the Figures.

#### **EXAMPLE 4**

##### **Mutagenesis Strategy from Clone Luc78-0B10 to Luc90-1B5**

Twenty-three oligonucleotides were prepared to change 28 positions to consensus. All of the oligonucleotides were tested individually using oligonucleotide directed mutagenesis with single stranded DNA from clone *luc78-0B10* as a template to determine which oligonucleotides gave an improvement in thermostability. Table 4 lists the mutagenic oligonucleotides.

Table 4

		OLIGO SYNTHESIS	
		NUMBER	SEQ ID NO.
5	A17 to T	6215	48
	M25 to L	6216	49
	S36 to P; remove <i>Nsi</i> I site	6217	50
	A101 to V, S105 to N	6218	51
	I125 to V	6219	52
10	K139 to Q	6220	53
	V145 to I	6221	54
	V194 to I	6222	55
	V203 to L, S204 to P	6231	56
	A216 to V	6232	57
15	A229 to Q	6233	58
	<b>M249 to T (reversion)</b>	<b>6234*</b>	59
	T266 to R, K270 to E	6235	60
	E301 to D	6236	61
	N333 to P, F334 to G	6237	62
20	R356 to K	6238	63
	I363 to V	6246	64
	A393 to P	6247	65
	R417 to H	6248	66
	G482 to V	6249	67
25	N492 to T	6250	68
	F499 to Y, S501 to A	6251	69
	L517 to V	6252	70
	F537 to L	6253	71

\*Note that oligonucleotide #6234 does not change a consensus position. This oligonucleotide causes a reversion of position 249 to the wild-type Ppe-2 codon. Although reversion of this position was shown to increase thermostability at 62°C, reversion of this position decreased light output.

Three oligonucleotide-directed mutagenesis experiments with clone *luc78-0B10* as a template were completed. The oligonucleotides for these experiments were divided in the following manner:

a. 6215, 6234, 6236, 6248 (found to give increased thermostability)

b. 6215, 6217, 6218, 6219, 6220, 6221, 6222, 6231, 6233, 6234, 6236, 6238, 6247, 6248, 6249, 6251, 6253 (found to be neutral or have increased thermostability).

c. All 23 oligonucleotides.

5           Selections from the three experiments listed above were screened with the robotic screening procedure (experiment 84, see Table 1) using *luc78-OB10* as a control. Selections from experiment 84 were recombined using the recombination mutagenesis procedure and then screened with the robotic screening procedure (experiment 85).

10           Single stranded DNA was prepared from three clones, *luc85-3E12*, *luc85-4F12*, *luc85-5A4*. The nucleotide sequence and inferred amino acid sequence of *luc85-4F12* are shown in Figures 40 and 41, respectively. These clones were used as templates for oligonucleotide-directed mutagenesis to improve codon usage. Positions were selected based upon a codon usage table  
15           published in Nucleic Acids Research, vol. 18 (supplement) 1990, page 2402. The table below lists oligonucleotides that were used to improve codon usage in *E. coli*.

Table 5

	Description	Oligo Synthesis #	SEQ ID NO.
	L7(tta-ctg), remove <i>Apa</i> I site	6258	72
	L29(tta-ctg)	6259	73
5	T42(aca-acc)	6260	74
	L51, L56(tta-ctg), L58(ttg-ctg)	6261	75
	L71(tta-ctg)	6262	76
	L85(ttg-ctg)	6263	77
	L95(ttg-ctg), L97(ctt-ctg)	6273	78
10	L113, L117(tta-ctg)	6274	79
	L151, L153(tta-ctg)	6275	80
	L163(ctc-ctg)	6276	81
	R187(cga-cgt)	6277	82
	L237(tta-ctg)	6279	83
15	R260(cga-cgc)	6280	84
	L285, L290(tta-ctg), L286(ctt-ctg)	6281	85
	L308(tta-ctg)	6282	86
	L318(tta-ctg)	6283	87
	L341(tta-ctg), T342(aca-acc)	6284	88
20	L380(ttg-ctg)	6285	89
	L439(tta-ctg)	6286	90
	L456(ctc-ctg), L457(tta-ctg)	6293	91
	T506(aca-acc), L510(cta-ctg)	6305	92
25	R530(aga-cgt)	6306	93

In the first experiment, the three templates listed above from experiment 85 were combined and used as a templates for oligonucleotide-directed mutagenesis. All of the oligonucleotides were combined in one experiment and clones resulting from oligonucleotide-directed mutagenesis were screened using the robotic screening procedure as experiment 88. There were a low percentage of luminescent colonies that resulted from this experiment, so another oligonucleotide-directed mutagenesis experiment was completed in which the oligonucleotides were combined in the following groups:

- a. 6258, 6273, 6280, 6286
- b. 6259, 6274, 6281, 6293
- c. 6260, 6275, 6282, 6294

d. 6261, 6276, 6283, 6305

e. 6262, 6277, 6284, 9306

f. 6263, 6279, 6285

5           It was discovered that samples from group b had a low number of  
luminescent colonies, and it was hypothesized that one of the oligonucleotides in  
group b was causing problems. Selections were made from all of the  
experiments with the exception of experiment b. Samples were then run through  
the robotic screening procedure (experiment 89). Selections from experiments  
10 88 and 89 were shuffled together with the recombination mutagenesis protocol  
and were then screened with the robotic screening procedure (experiment 90).

#### Materials and Methods

##### A. Mutagenesis Protocol

15           The mutant luciferases disclosed herein were produced via random  
mutagenesis with subsequent *in vivo* screening of the mutated genes for a  
plurality of characteristics including light output and thermostability of the  
encoded luciferase gene product. The mutagenesis was achieved by generally  
following a three-step method:

- 20 1. Creating genetic diversity through random mutagenesis. Here, error-  
prone PCR of a starting sequence was used to create point mutations in  
the nucleotide sequence. Because error-prone PCR yields almost  
exclusively single point mutations in a DNA sequence, a theoretical  
maximum of 7 amino acid changes are possible per nucleotide mutation.  
25 In practice, however, approximately 6.1 amino acid changes per  
nucleotide is achievable. For the 550 amino acids in luciferase,  
approximately 3300 mutants are possible through point mutagenesis.
2. Consolidating single point mutations through recombination  
mutagenesis. The genetic diversity created by the initial mutagenesis is  
30 recombined into a smaller number of clones by sPCR. This process not  
only reduces the number of mutant clones, but because the rate of  
mutagenesis is high, the probability of linkage to negative mutations is  
significant. Recombination mutagenesis unlinks positive mutations from

negative mutations. The mutations are “re-linked” into new genes by recombination mutagenesis to yield the new permutations. Then, after re-screening the recombination mutants, the genetic permutations that have the “negative mutations” are eliminated by not being selected.

5 Recombination mutagenesis also serves as a secondary screen of the initial mutants prepared by error-prone PCR.

3. Broadening genetic diversity through random mutagenesis of selected codons. Because random point mutagenesis can only achieve a limited number of amino acid substitutions, complete randomization of selected  
10 codons is achieved by oligonucleotides mutagenesis. The codons to be mutated are selected from the results of the preceding mutagenesis processes on the assumption that for any given beneficial substitution, other alternative amino acid substitutions at the same positions may produce even greater benefits. The positions to be mutated are identified  
15 by DNA sequencing of selected clones.

#### B. Initial mutagenesis experiments

Both the N-terminus and the C-terminus of the starting sequence were modified by oligonucleotide-directed mutagenesis to optimize expression and  
20 remove the peroxisomal targeting sequence. At the N-terminus, nine bases downstream of the initiation codon were randomized. At the C-terminus, nine bases upstream of the termination codon were randomized. Mutants were analyzed using an *in vivo* screen, resulting in no significant change in expression.

Six clones from this screen were pooled, and used to mutate the codons  
25 for seven cysteines. These codons were randomized using oligonucleotide-directed mutagenesis, and the mutants were screened using the robotic screening procedure. From this screen, fifteen clones were selected for directed evolution.

#### C. Generating and Testing Clones

30 Several very powerful and widely known protocols are used to generate and test the clones of the present invention. Unless noted otherwise, these laboratory procedures are well known to one of skill in the art. Particularly noted as being well known to the skilled practitioner is the polymerase chain

reaction (PCR) devised by Mullis and various modifications to the standard PCR protocol (error-prone PCR, sPCR, and the like), DNA sequencing by any method (Sanger or Maxam & Gilbert's methodology), amino acid sequencing by any method (e.g., the Edman degradation), and electrophoretic separation of  
5 polynucleotides and polypeptides/proteins.

#### D. Vector Design

A preferred vector (pRAM) (see Figure 20) used for the mutagenesis procedure contains several unique features that allow for the mutagenesis  
10 strategy to work efficiently:

The pRAM vector contains a filamentous phage origin, *fl*, which is necessary for the production of single-stranded DNA.

Two *Sfi*I sites flank the gene. These sites were designed by so that the gene to be subcloned can only be inserted in the proper orientation.

15 The vector contains a *tac* promoter.

Templates to be used for oligonucleotide mutagenesis contain a 4 base-pair deletion in the *bla* gene which makes the vector ampicillin-sensitive. The oligonucleotide mutagenesis procedure uses a mutant oligonucleotide as well as an ampicillin repair oligonucleotide that restores function to the *bla* gene. This  
20 allows for the selection of a high percentage of mutants. (If selection is not used, it is difficult to obtain a high percentage of mutants.)

#### E. Uses of Luciferases

The mutant luciferases of the present invention are suitable for use in any  
25 application for which previously known luciferases were used, including the following:

ATP Assays. The greater enzyme stability means that reagents designed for detection of ATP have a greater shelf-life and operational-life at higher temperatures (e.g., room temperature). Therefore, a method of detecting ATP  
30 using luciferases with increased thermostability is novel and useful.

Luminescent labels for nucleic acids, proteins, or other molecules.

Analogous to advantages of the luciferases of the present invention for ATP assays, their greater shelf-life and operational-life is a benefit to the reliability

and reproducibility of luminescent labels. This is particularly advantageous for labeling nucleic acids in hybridization procedures where hybridization temperatures can be relatively high (e.g., greater than 40°C). Therefore, a method of labeling nucleic acids, proteins, or other molecules using luciferases of the present invention is novel and useful.

Genetic reporter. In the widespread application of luciferase as a genetic reporter, where detection of the reporter is used to infer the presence of another gene or process of interest, the increased thermostability of the luciferases provides less temperature dependence of its expression in living cells and in cell-free translations and transcription/translation systems. Therefore, a method using the luciferases of the present invention as genetic reporters is novel and useful.

Enzyme immobilization. Enzymes in close proximity to physical surfaces can be denatured by their interaction with that surface. The high density immobilization of luciferases onto a surface to provide strong localized luminescence is improved by using thermostable luciferases. Therefore, a method of immobilizing luciferases onto a solid surface using luciferases of the present invention is novel and useful.

Hybrid proteins. Hybrid proteins made by genetic fusion genes encoding luciferases and of other genes, or through a chemical coupling process, benefit by having a greater shelf-life and operational-life. Therefore, a method of producing hybrid proteins through genetic means or chemical coupling using the luciferases of the present invention is novel and useful.

High temperature reactions. The light intensity of a luciferase reaction increases with temperature until the luciferase begins to denature. Because the use of thermostable luciferases allows for use at greater reaction temperatures, the luciferases of the present invention are novel and useful for performing high temperature reactions.

Luminescent solutions. Luminescence has many general uses, including educational, demonstrational, and entertainment purposes. These applications benefit from having enzymes with greater shelf-life and operational-life. Therefore, a method of making luminescent solutions using the luciferases of the present invention is novel and useful.



F. Firefly luciferase

The firefly luciferase gene chosen for directed evolution was *LucPpe2* isolated from *Photuris pennsylvanica*. The luciferase was cloned from fireflies collected in Maryland by Wood et al. and later was independently cloned by Dr. Leach using fireflies collected in Oklahoma (Ye et al., 1997). A mutant of this luciferase (T249M) was made by Wood et al. and used in the present invention because it produced approximately 5-fold more light when expressed in colonies of *E. coli*.

Overview of Evolution Process: Directed evolution was achieved through a recursive process, each step consisting of multiple cycles of 1) creating mutational libraries of firefly luciferase followed by 2) screening the libraries to identify new mutant clones having a plurality of desired enzymological characteristics.

To begin the process, three mutational libraries were created using error-prone PCR (Fromant *et al.*, 1995). Each library was screened first by visual evaluation of luminescence in colonies of *E. coli* (Wood and De Luca, 1987), and then by quantitative measurements of enzymological properties in *E. coli* cell lysates. Approximately 10,000 colonies were examined in the visual screen, from which 704 were selected for quantitative analysis. From each quantitative screen 18 clones were selected. The three sets of 18 clones each were pooled together, and a new mutational library was created using DNA shuffling to generate intragenetic recombinations (sPCR; Stemmer, 1994). The results were screened to yield another set of 18 clones. The entire process was completed by combining this set of 18 clones with 18 clones from the previous round of evolution, creating another mutational library by DNA shuffling, and screening as before.

Screening method: In the qualitative visual screen, colonies were selected only for their ability to sustain relatively bright luminescence. The thermal stability of the luciferase within the colonies of *E. coli* was progressively challenged in successive rounds of evolution by increasing the temperature of the screen. The selected colonies were inoculated into wells of 96-well plates each containing 200  $\mu$ l of growth medium.

In the quantitative screens, lysates of the *E. coli* cultures were measured for 1) luminescence activity, 2) enzyme stability, 3) sustained enzymatic turnover, and 4) substrate binding.

“Luminescence activity” was measured as the ratio of luminescence  
5 intensity to the optical density of the cell culture.

“Enzyme stability” was determined by the rate of activity loss from cell lysates over 10 hours. In successive rounds of evolution the incubation temperature of the lysates was increased.

“Sustained enzymatic turnover” was determined by the rate of  
10 luminescence loss of a signal enzymatic reaction over 10 hours at room temperature.

“Substrate binding” was determined by the relative activity of the lysate when assayed with diluted substrate mixtures. Of these four parameters, the highest priority for selection was placed on thermostability.

15        Robotic Automation: Robotic automation was used in the quantitative screens to accurately perform the large number of required quantitative assays on the cultured cells. Overnight cultures were first diluted into fresh medium and grown for 3 hours to produce cultures in mid-log phase growth. The optical densities of each culture was then measured, and aliquots of the cultures were  
20 lysed by freeze/thaw and lysozyme. The resulting lysates were further diluted before analysis and incubated at elevated temperatures. Luminescence was measured from aliquots of the diluted lysates, taken at various times, and measured under various conditions as prescribed by the analytical method (see Example 2). Computer analysis of this data yielded the quantitative selection  
25 criteria described herein.

Summary of evolutionary progression: After mutagenesis of the N- and C-termini, and randomization of the cysteine codons, a pool of 15 clones was subjected to two rounds of directed evolution as described herein. Five of the 18 clones resulting from this process were sequenced to identify mutations. One of  
30 these clones designated, Luc49-7C6, was chosen for more detailed analysis and further mutagenesis. This clone contained 14 new amino acid substitutions compared to the luciferase Luc[T249M].

To assess the potential for other amino acid replacements at the sites of these substitutions, oligonucleotide-directed mutagenesis was used to randomize these codons. The resulting clones were screened as described herein, and 18 selected clones were used to initiate two new rounds of directed evolution. Of  
5 the 18 clones resulting from this second set of rounds, the clone designated Luc78-0B10 was chosen for additional study and mutagenesis. This clone encoded a luciferase that contained 23 new amino acid substitutions compared to Luc[T249M].

Using oligonucleotide directed mutagenesis with Luc78-0B10 as the  
10 template, codons were selected for substitution to consensus amino acids previously known among beetle luciferases. Selections from this mutagenesis experiment were shuffled together and three clones, determined to be the most stable were then used as templates for oligonucleotide mutagenesis to improve codon usage in *E. coli*. A clone designated Luc90-1B5 selected from this  
15 experiment, contained 34 amino acid substitutions relative to Luc[T249M] (see Figures 42 and 43 for the nucleotide sequence and inferred amino acid sequence of *luc90-1B5*, and Figures 44 and 45 for the nucleotide sequence encoding and the inferred amino acid sequence of Luc[T249M]). Out of 25 codons selected for change to consensus amino acids, 11 were replaced in the clone designated  
20 Luc90-1B5. Only five out of the 30 positions that were selected for improved codon usage were substituted and had little effect on enzyme expression.

Protein purification: Four mutants that are described herein (Luc[T249M], Luc49-7C6, Luc78-0B10, and Luc90-1B5) were purified using a previously published procedure (Hastings et al., 1996).

25 Enzymological characterization: Purified proteins were diluted in 25 mmol/L HEPES pH 7.8, 150 mmol/L NaCl, 0.1 mmol/L EDTA, 1 mg/ml BSA. Enzyme stability was determined from diluted proteins incubated at different temperatures, and aliquots were removed at different time points. A linear regression of the natural log of the luminescence and time was calculated.  
30 Half-life was calculated as the  $\ln(0.5)/\text{slope}$  of the regression.

# G. PCR Mutagenesis Protocol (Random Mutagenesis)

## PCR mutagenesis reactions

1. Prepare plasmid DNA from a vector containing the gene of interest, estimate DNA concentration from a gel.
  2. Set up two 50  $\mu$ l reactions per group:
- There are three groups of mutagenic conditions using different skewed nucleotide concentrations.

The conditions listed herein yield in the range of from 8-10% wild-type Luc colonies after subcloning phenotypic for each generated parent clone. The rate of mutagenesis is estimated by the number of luminescent colonies that are present after mutagenesis. Based upon results of clones mutated in the range of 8-10%, it was determined that this level of mutagenesis produces on average approximately 2-3 amino acid changes per gene. If the mutagenesis rate is selected so that on average there is one amino acid change per gene, then on average 50% of the clones will have no mutations. (Bowie et al., 1990).

For the master mix: add all components (see Table 6) except polymerase, vortex, spin briefly, add polymerase, and mix gently.

**Table 6**

Component	A to T/T to A	A to C/T to G	G to A/C to T
dATP	0.3 mM	0.1 mM	0.25 mM
dCTP	2.75 mM	4 mM	1 mM
dGTP	0.06 mM	0.02 mM	0.05 mM
dTTP	0.625 mM	0.3 mM	0.6 mM
<sup>++</sup> pRAMtailUP	0.4 pmol/ $\mu$ l	0.4 pmol/ $\mu$ l	0.4 pmol/ $\mu$ l
<sup>++</sup> pRAMtailDN	0.4 pmol/ $\mu$ l	0.4 pmol/ $\mu$ l	0.4 pmol/ $\mu$ l
*Taq Polymerase	1 U/ $\mu$ l	1 U/ $\mu$ l	1 U/ $\mu$ l
$^{\circ}$ MgCl <sub>2</sub>	6.77 mM	5.12 mM	2.7 mM
$^{\circ}$ MnCl <sub>2</sub>	0.5 mM	0.5 mM	0.3 mM
DNA	50 ng total	50 ng total	50 ng total
10X PCR buffer	1X	1X	1X
Autoclaved nanopure water	To 50 $\mu$ l	To 50 $\mu$ l	To 50 $\mu$ l

\* Taq Polymerase is purchased from Perkin Elmer (N808-0101).

$^{\circ}$  MnCl<sub>2</sub> and MgCl<sub>2</sub> are made fresh from 1 M stocks. The stocks are filter sterilized and mixed with sterile water to make the 10 mM and 25 mM stocks which are then stored in Polystyrene Nalgene containers at 4°C.

<sup>++</sup> pRAMtailUP: 5'-gtactgagacgacgccagcccaagcttaggcctgagtg-3' (SEQ ID NO:38); pRAMtailDN: 5'-ggcatgagcgtgaactgactgaactagcggccgcccag-3' (SEQ ID NO:39)

5    10X PCR polymerase buffer:

- 100 mM Tris-HCl pH 8.4 from 1 M stock
- 500 mM KCl
- Primers are diluted from a 1 nmol/μl stock to a 20 pmol/μl working stock.

10        Cycle in thermal cycler: 94°C for 1 minute (94°C for 1 minute, 72°C for 10 minutes) 10X

3. Purify reaction products with Wizard PCR purification kit (Promega Corporation, Madison, Wisconsin, part#A718c):

- transfer PCR reaction into a new tube containing Promega 100  
15                    μl Direct Purification buffer (Promega part#A724a)
- add 1 ml of Wizard PCR Purification Resin (Promega  
part#A718c) Promega and incubate at room temperature  
for 1 minute
- pull resin through Wizard minicolumn
- 20        - wash with 80% ethanol
- spin in microcentrifuge to remove excess ethanol
- elute into 50 μl sterile nanopure water (allow water to remain on  
column for at least 1 minute)

*Amplification<sup>1</sup> Of Mutagenesis Reaction*

1. Set up five 50  $\mu$ l reactions (see Table 7) per group.

**Table 7**

	Components	Concentration	Amount in 50 $\mu$ l	Final concentration
5	dATP	10 mM	1 $\mu$ l	0.2 mM
	dCTP	10 mM	1 $\mu$ l	0.2 mM
	dGTP	10 mM	1 $\mu$ l	0.2 mM
	dTTP	10 mM	1 $\mu$ l	0.2 mM
	+pRAM18UP	20 pmol/ $\mu$ l	1 $\mu$ l	0.4 pmol/ $\mu$ l
10	+pRAM19DN	20 pmol/ $\mu$ l	1 $\mu$ l	0.4 pmol/ $\mu$ l
	Pfu polymerase	2 U/ $\mu$ l	1 $\mu$ l	0.04 $\mu$ / $\mu$ L
	°10X buffer	10X	5 $\mu$ l	1X
	DNA		10 $\mu$ l	
	Water		24.6 $\mu$ l	

15

- To master mix: add all components, except polymerase, vortex, spin briefly, add polymerase, mix gently.

- ° 10X reaction buffer for Native Pfu polymerase contains 20 mM  $MgCl_2$ , so no additional  $MgCl_2$  needs to be added

20

- + primers:

pRAM18UP -5'-gtactgagacgacgccag-3' (SEQ ID NO:40)

pRAM19DN -5'-ggcatgagcgtgaactgac-3' (SEQ ID

NO:41)

- Cycling conditions: 94°C for 30 seconds (94°C for 20 seconds,

25

65°C for 1 minute, 72°C for 3 minutes) 25X

(Perkin-Elmer Gene Amp® PCR System 2400)

2. Load 1  $\mu$ l on a gel to check amplification products

---

<sup>1</sup> This amplification step with Pfu polymerase was incorporated for 2 reasons: (a) To increase DNA yields for the production of large numbers of transformants. (b) To reduce the amount of template DNA that is carried over from the mutagenic PCR reaction: (Primers for the second amplification reaction are nested within the mutagenic primers. The mutagenic primers were designed with non-specific tails of 11 and 12 bases respectively for the upstream and downstream primers. The nested primers will amplify DNA that was previously amplified with the mutagenic primers, but cannot amplify pRAM template DNA.)

3. Purify amplification reaction products with Wizard PCR purification kit (Promega Corporation, part#A718c):

- transfer PCR reaction into a new tube containing 100 µl Direct Purification buffer (Promega, Part#A724a)
- 5       - add 1 ml of Wizard PCR Purification Resin (Promega Part#A718c) and incubate at room temperature for 1 min
- pull resin through Wizard minicolumn
- wash with 80% Ethanol
- spin in microcentrifuge to remove excess Ethanol
- 10       - elute with 88 µl sterile nanopure water (allow water to remain on column for at least 1 min)

Subcloning of amplified PCR mutagenesis products

1. Digest the DNA with *Sfi* I as follows:
  - 2 µl *Sfi* I (Promega Part #R639a)
  - 15       - 10 µl 10X buffer B (Promega Part #R002a)
  - 88 µl of DNA from Wizard PCR prep (see step 3 above)
  - mix components and overlay with 2 drops of mineral oil; incubate at 50°C for 1 hour
2. Remove salts and *Sfi* I ends with Wizard PCR purification as described herein, and elute into 50 µl sterile nanopure water
- 20       3. Ligation into pRAM (+/r) backbone (set up 4 ligations per group):
  - 0.025 pmol pRAM backbone
  - 0.05 pmol insert (usually in the range of 6 to 12 µl of insert)
  - 1 µl of T4 DNA Ligase (Promega part M180a)
  - 25       - 2 µl of 10X ligase buffer (Promega part C126b, divide into 25 µl aliquots, do not freeze/thaw more than twice)
  - water to 20 µl
  - ligate for 2 hours at room temperature
  - heat reactions for 15 minutes at 70°C to inactivate ligase

30   Transformation and plating

1. Butanol precipitate samples to remove excess salts (n-Butanol from Sigma, St. Louis, Missouri, part #BT-105):

(if ethanol precipitation is used instead of butanol, a wash with 70% ethanol as needed. Excess salt will cause arcing during the electroporation which causes the reaction to fail.)

- 5                   - add water to 50  $\mu$ l  
                  - add 500  $\mu$ l of n-butanol  
                  - mix until butanol/ligation mix is clear and then spin for 20 min  
                                at room temperature  
                  - drain butanol into waste container in fume hood
- 10               - resuspend in 12  $\mu$ l water, spin 30 sec at full speed
2.               Preparation of cell/DNA mix (set up 4 transformations plus one  
                  with reference clone DNA):
- while DNA is precipitating, place electroporation cuvettes on ice  
                  - fill 15 ml Falcon snap-cap tubes with 3 ml S.O.C. medium and
- 15                               place on ice
- thaw JM109 electrocompetent cells on ice (50  $\mu$ l per ligation  
                                reaction)
- pipette 10  $\mu$ l of the bottom layer from step 1 (or 0.5  $\mu$ l ref.clone  
                                DNA) into competent cells
- 20               (small amounts of butanol carry-over do not adversely effect the  
                                transformation efficiency)
- place cell/DNA mix on ice
3.               Electroporation:
- carry tubes, cuvettes, and cell/DNA mix on ice to
- 25                               electroporation device
- pipette cell-DNA mix into a cuvette and zap. Instrument
- settings:
- Cuvette gap: 0.2 cm
- Voltage: 2.5 kV
- 30               Capacitance: 25  $\mu$ F
- Resistance: 200 Ohms
- Time constant: 4.5 msec



- pipette 1 ml SOC (contains KCl; media prep #KCLM) into  
cuvette, quickly pour into recovery tube (transformation  
efficiency is reduced if cells are allowed to sit in cuvette)
- place the recovery tube on ice until all samples are processed
- 5 - allow the cells to recover at 37°C for 30-60 minutes
- plate on LB + amp plates with nitrocellulose filters  
(# of colonies is about 20% higher if cells recover 60 minutes,  
possibly due to cell replication.)  
(Best colony density for screening is 500 per plate. For the  
10 current batch of cells plate about 500 to 750 µl)

#### H. Recombination Mutagenesis Protocol or DNA shuffling

##### *DNase I digestion of plasmid DNA*

1. Prepare 2% low melting point gel
 

15 - use 0.8 g agarose in 40 ml (NuSieve #50082)

- use large prep comb

- make sure it is solidified prior to digesting
2. Prepare 4 µg of pooled plasmid DNA for digest
3. Prepare 1 U/µl DNase I dilution on ice according to the table  
20 below:

**Table 8**

25	DNase I <sup>+</sup>	0.74 µl
	10X DnaseI buffer	10 µl
	1% gelatin*	10 µl
	Water to 100 µl	

<sup>+</sup> DNase I from Sigma (D5791)

\* Gelatin was added to keep the DNase I from sticking to the walls of the tubes.

- 30 This dilution can be kept on ice for at least 30 min without loss in  
activity.

#### 4. Digest (set up at room temperature):

- prepare two digests with 1.0 U and 1.5 U DNase I per 100 µl  
35 reaction:

- 7.8)
- 10  $\mu$ l of 10X DNase I buffer (500 mM Tris, 10 mM  $\text{MgCl}_2$  pH
  - x  $\mu$ l DNA ( 2  $\mu$ g of pooled plasmid DNA from step 2)
  - 1 or 1.5  $\mu$ l of the 1 U/ $\mu$ l enzyme dilution
  - 5 - sterile nanopure water to 100  $\mu$ l
  - incubate at room temperature for 10 minutes
  - stop reaction by addition of 1  $\mu$ l of 100 mM CDTA

Purification from agarose gel

1. Run DNase digested fragments on gel
- 10 - add 10  $\mu$ l of 10X loading buffer to each DNase I digest
- load all on a 2% Low melting point agarose gel
- run about 30 min at 120-150 V
- load pGEM DNA marker in middle lane
2. Isolate fragments
- 15 - cut out agarose slice containing fragments in the size range of  
600-1000 bp using a razor blade
- cut into pieces that weigh about 0.3 g
- melt the gel slices at 70°C
- add 300  $\mu$ l of Phenol (NaCl/Tris equilibrated) to the melted
- 20 agarose, vortex for about 1 minute at max speed
- spin for 10 min at 4°C
- remove the top layer into a tube containing an equal volume of  
Phenol/Chloroform/Isoamyl (saturated with 300 mM NaCl  
/100 mM Tris pH 8.0), vortex and centrifuge for 5 minutes
- 25 at RT
- remove the top layer into a tube containing chloroform and  
vortex and centrifuge.
- remove the top layer into a tube with 2 vol. of 95% cold  
Ethanol; place in -70°C freezer for 10 min (no additional
- 30 salts are needed because of the High Salt Phenol)
- spin at 4°C for 15 minutes.
- wash with 70% Ethanol, drain and air dry for ~10 min
- resuspend in 25 to 50  $\mu$ l of sterile nanopure water

70

- store at -70°C until ready for use

### Assembly reaction

Set up 4 reactions (see Table 9) and pool when completed.

5

**Table 9**

	Component	Concentration	Amount in $\mu$ l	Final concentration
	dATP	10 mM	1	200 $\mu$ M
	dCTP	10 mM	1	200 $\mu$ M
	dGTP	10 mM	1	200 $\mu$ M
10	dTTP	10 mM	1	200 $\mu$ M
	DNA*	1-10 ng	5	
	Tli	3 U/ $\mu$ l	0.4	0.24 U/ $\mu$ l
	10X Thermo buffer	10X	5	1X
	MgCl <sub>2</sub>	25 mM	4	2 mM
15	gelatin	1%	5	0.1%
	water		To 50 $\mu$ l	

\* Because the DNA used for this reaction has been fragmented, it is difficult to estimate a concentration. The easiest way is to load 5  $\mu$ l of the DNaseI digested DNA to an agarose gel and run the gel until the dye enters the wells (1-2 min). Fragments from a typical 2  $\mu$ g DNA digest which were resuspended in 100  $\mu$ l of water give a DNA concentration of about 1 to 10 ng/ $\mu$ l.

Cycling conditions: 94°C for 30 seconds (94°C for 20 seconds, 65°C for 1 minute, 72°C for 2 minutes) 25X

### Amplification of assembly

Usually 5 amplification reactions (see Table 10) will produce enough DNA for a full 8 plate robotic run.

30

**Table 10**

	Component	Concentration	Amount in $\mu$ l	Final concentration
5	dATP	10 mM	1	200 $\mu$ M
	dCTP	10 mM	1	200 $\mu$ M
	dGTP	10 mM	1	200 $\mu$ M
	dTTP	10 mM	1	200 $\mu$ M
	pRAMtailUP*	20 pmol/ $\mu$ l	2	0.8 pmol/ $\mu$ l
10	pRAMtailDN*	20 pmol/ $\mu$ l	2	0.8 pmol/ $\mu$ l
	Pfu native polymerase <sup>+</sup>	2 U/ $\mu$ l	1	0.04 U/ $\mu$ l
	10X native Pfu buffer <sup>o</sup>	1X	5	1X
	DNA	1-10 ng	5	
	water		water to 50 $\mu$ l	

\* Note that the concentration of primers is twice as high as in a typical amplification reaction.

<sup>o</sup> The Pfu 10X buffer contains 20 mM  $MgCl_2$ , so it is not necessary to add  $MgCl_2$ .

<sup>+</sup> Pfu polymerase is ordered from Stratagene part #600135. Cycling conditions: 94°C for 30 seconds (94°C for 20 seconds, 65°C for 1 minute, 72°C for 3 minutes) 25x

#### Subcloning of assembly amplification

Purify amplification products with Wizard PCR purification:

- pool 5 amplification reactions
- transfer into a new tube that contains 100  $\mu$ l of Direct Purification buffer
- add 1 ml of Wizard PCR Purification Resin, incubate at RT for 1 minute
- pull Resin though Wizard minicolumn
- wash with 80% ethanol and spin in microcentrifuge to remove excess ethanol
- elute with 88  $\mu$ l of sterile nanopure water (allow water to remain on column for at least 1 minute)

2. Digest with *Sfi* I:

- 2  $\mu$ l *Sfi* I
- 10  $\mu$ l 10X buffer B
- 88  $\mu$ l of DNA from Wizard PCR prep

- mix components and overlay with 2 drops of mineral oil;  
incubate at 50°C for 1 hour

3. Band isolation:

5 Sometimes after amplification of the assembly reaction a band that is smaller than the gene-sized fragment is produced. This small fragment has been shown to subclone about 10-fold more frequently than the gene sized fragment if the sample is not band isolated. When this contaminating band is present, it is necessary to band isolate after *Sfi* I digestion.

- 10
- load the DNA to a 0.7% agarose gel
  - band isolate and purify with the Gene Clean kit from Bio 101
  - elute DNA with 50 µl sterile nanopure water, check concentration on gel (This type of purification with standard agarose produced the highest number of
- 15 transformants after subcloning. Other methods tried: Low melt with Phenol chloroform, Gene clean with low melt, Wizard PCR resin with standard agarose, Pierce Xtreme spin column with Low melt (did not work with standard agarose)).

- 20
4. Ligate into pRAM [+/-] backbone: (See ligation and transformation protocol above)

Large scale preparation of pRAM backbone

- 25
1. Streak an LB amp plate with pRAMMCS [+/-] (This vector contains a synthetic insert with a *Sac* II site in place of a gene. This vector contains the new ribosome binding site, but it will be cut out when the vector is digested with *Sfi* I.
  2. Prepare a 10 ml overnight culture in LB supplemented with amp.
  3. The next day inoculate 1 L of LB supplemented with amp and
- 30 grow for 16-20 hours.
4. Purify the DNA with the Wizard Maxi Prep kit. (Promega #A7270) (use 4 preps for 1 L of cells)

5. Digest the Plasmid with *Sfi* I. (Use 5 U per microgram) Overlay with mineral oil and digest for at least two hours.
6. Ethanol precipitate to remove salts. Resuspend in water.
7. Digest with *Sac* II for 2 hours. (Keep digest volume to 2 ml or less). It is possible that part of the plasmid could be partially digested. If the vector is cut with an enzyme that is internal to the two *Sfi* I sites, it will keep the partially digested fragments from joining in a ligation reaction.
8. Load entire digest onto a column (see 9). The volume of the sample load should not be more than 2 ml. If it is it will be necessary to ethanol precipitate.
9. The column contains Sephacryl S-1000 and is stored with 20% ethanol to prevent bacterial contamination. Prior to loading the sample the column must be equilibrated with cold running buffer for at least 24 hours. If the column has been sitting more than a couple of months it may be necessary to empty the column, equilibrate the resin 3-4 washes in cold running buffer, and then re-pour the column. After the column is poured it should be equilibrated overnight so that the resin is completely packed.
10. Collect fractions of about 0.5 ml. Typically the DNA comes off between fractions 25 and 50. Load a 5  $\mu$ l aliquot from a range of fractions to determine which fractions contain the backbone fragment. The small insert fragment will start to come off the column before all of the backbone is eluted, so it will be necessary to be conservative when fractions are pooled. For this reason typically 40-60% of the DNA is lost at this step.
11. Pool the fractions that contain the backbone.
12. Ethanol precipitate the samples. Resuspend in a volume that produces about 10-50 ng/ $\mu$ l.

13. Store at -70°C.

Column running buffer: (store at 4°C)

5 mM EDTA

5 100 mM NaCl

50 mM Tris-HCL pH 8.0

10 µg/ml tRNA (R-8759, Sigma)

# I. Oligonucleotide Mutagenesis

10 Prepare Ampicillin-sensitive single stranded DNA of the template to be mutated. Design a mutagenic primer that will randomly generate all possible amino acid codons.

*Mutagenesis reaction:*

15

**Table 11**

	Component	Final concentration
	Single Stranded Template	0.05 pmol
	Mutagenic Oligonucleotide	1.25 pmol
	Ampicillin Repair Oligo (Promega q631a)	0.25 pmol
20	10X annealing buffer*	1X
	Water to 20 µl	

\*10X Annealing buffer:

-200 mM Tris-HCl, pH 7.5

25 -100 mM MgCl<sub>2</sub>

-500 mM NaCl

Heat reaction at 60°C for 15 minutes and then immediately place on ice.

*Synthesis reaction:*

**Table 12**

	Component	Amount
5	Water	5 $\mu$ l
	10X synthesis buffer*	3 $\mu$ l
	T4 DNA Polymerase (Promega m421a)	1 $\mu$ l (10 Units)
	T4 DNA Ligase (Promega 180a)	1 $\mu$ l (3 Units)

10 \*10X Synthesis buffer

100 mM Tris-HCl, pH 7.5

5 mM dNTPs

10 mM ATP

20 mM DTT

15 Incubate at 37°C for 90 minutes.

Transform into Mut-S strain BMH 71-18 (Promega strain Q6321)

-Place Synthesis reaction in a 17X100 mm tube.

-Add BMH 71-18 competent cells that have been thawed on ice to synthesis reaction.

20 -Incubate on ice for 30 min

-Heat Shock cells at 42°C for 90 seconds.

-Add 4 ml of LB medium and grow cells at 37°C for 1 hour. Add Ampicillin to a final concentration of 1.25 ug/ml and then grow overnight at 37°C.

25 Isolate DNA with Wizard Plus Purification system (Promega a7100)

Transform isolated DNA into JM109 electrocompetent cells and transform onto LB Ampicillin plates.

#### J. Screening procedure

30 JM109 clones (from a transformation reaction) are plated onto nitrocellulose filters placed on LB amp plates at a screening density of about 500 colonies per plate.



As listed in the Random Mutagenesis procedure, approximately 10% of the clones to be selected will have to be as stable as the same sequenced or better than source. Or stated another way, about 50 colonies per plate will be suitable for selection. There are 704 wells available for a full eight plate robotic run, so  
5 at least 15 LB amp plates will be needed for a full robotic run.

After overnight growth at 37°C the plates containing the transformants are removed from the incubator and placed at room temperature.

The nitrocellulose filter is lifted on one side and 500 µl of 10 mM IPTG is added to each of the plates. The filter is then placed back onto the plate to  
10 allow diffusion of the IPTG into the colonies containing the different mutant luciferase genes. The plates are then incubated for about 4 hours at room temperature.

One (1) ml of a solution contains 1 mM luciferin and 100 mM sodium citrate is pipetted onto a slide warmer that is set at 50°C. A nitrocellulose filter  
15 that contains mutant luciferase colonies and has been treated with IPTG is then placed on top of the luciferin solution. After several minutes, the brightest colonies are picked with tooth picks which are used to inoculate wells in a microtiter plate that contain M9- minimal media with 1% gelatin.

After enough colonies are picked to 8 microtiter plates, the plates are  
20 placed in an incubator at 350 rpm at 30°C incubation and are grown overnight.

In the morning the overnight plates are loaded onto the robot and the cell dilution procedure is run. (This procedure dilutes the cultures 1:10 into induction medium). The new plates are grown for 3 hours at 350 rpm at 30°C.

After growth, the plates are loaded to the robot for the main assay  
25 procedure.

Minimal Media:

6 g/Liter Na<sub>2</sub>HPO<sub>4</sub>  
3 g/Liter KH<sub>2</sub>PO<sub>4</sub>  
0.5 g/Liter NaCl  
30 1 g/Liter NH<sub>4</sub>Cl  
2 mM MgSO<sub>4</sub>  
0.1 mM  
1 mM Thiamine-HCl

0.2% glucose  
 12 µg/ml tetracycline  
 100 µg/ml ampicillin

- 5       \*Overnight media contains 1% gelatin  
          \*Induction media contains 1 mM IPTG and no gelatin.

S.O.C. Media:

- 10 mM NaCl  
                   -2.5 mM KCl  
 10               -20 mM MgCl<sub>2</sub>  
                   -20 mM glucose  
                   -2% bactotryptone  
                   -0.5% yeast extract

15    Summary of Exemplary Evolutionary Progression

1.     Start with *LucPpe2*[T249M]
2.     Mutate 3 amino acids at N- and C-termini
3.     Mutate 7 cysteines
4.     Perform two iterations of evolution → *Luc49-7C6*
- 20    5.     Mutagenesis of altered codons (9)
6.     Two iterations of evolution → *Luc78-0B10*
7.     Mutagenesis of consensus codons (28)
8.     Mutagenesis of codon usage (24) → *Luc90-1B5*

25    One Iteration of Recursive Process

1.     1 clone → 3 libraries using error-prone PCR
  - 3 x Visual screen (about 10,000 clones each)
  - 3 x Quantitative screen (704) clones each)
2.     3 x 18 clones → library using sPCR
  - 30    • Visual screen (about 10,000 clones)
  - Quantitative screen (704 clones)
3.     18 + 18 → library using sPCR
  - Visual screen (about 10,000 clones)

- Quantitative screen (704 clones)

4. Output: 18 clones

5

### **EXAMPLE 5**

#### **Mutagenesis Strategy from Clone Luc90-1B5 to Luc133-1B2 and Luc146-1H2**

Upon storage, luciferin degrades and the degradation products inhibit luciferase. The production of inhibitors causes an apparent instability in the reagent containing both luciferase and luciferin. There are two ways to reduce this problem: 1) Store the luciferin and luciferase at pH 5.5-6.0 to reduce the rate of luciferin degradation, and/or 2) Evolve an enzyme that is resistant to the luciferin degradation products.

LucPpe2 mutants that were evolved after clone Luc90-1B5 were evolved to be more stable at low pH and have resistance to luciferin degradation products. These mutant enzymes are useful, for example, in an ATP detection kit. One embodiment of such a kit comprises a mixture of luciferin and luciferase. A luminescent reaction occurs when a sample comprising ATP is added to the mixture.

Three populations of random mutants were produced using clone Luc90-1B5 as a template. These three populations were screened on the robot as experiments 114, 115, and 117. Robotic screens for experiments 114, 115, 116, 117, 118, 119, and 122 were completed as described previously except that buffer C was prepared with citrate buffer pH 4.5 instead of HEPES buffer pH 7.8, and the assay reagent was prepared with HEPES pH 7.1 with 10  $\mu$ M ATP instead of Tricine pH 8.0 and 175  $\mu$ M ATP. These screening conditions were biased to select clones that have increased retention of luminescence activity over time at pH 4.5 at 48°C and increased luminescence activity when assayed at pH 7.1 with 10  $\mu$ M ATP. Seventeen clones from experiment 114, seven clones from experiment 115, and ten clones from experiment 116 were shuffled together using sPCR and selected mutants from this screen were run on the robot as experiment 117. Eighteen clones were selected from experiment 117.

The clone that was determined to have the most improved characteristics (increased retention of luminescence activity over time at pH 4.5 and 48°C and increased luminescence activity when assayed at pH 7.1 with 10  $\mu$ M ATP) was clone Luc117-3C1 and it was selected as a template for random mutagenesis.

- 5 Two populations of random mutants were screened and then run on the robot as experiments 118 and 119. Seven clones from experiment 118 and five clones from experiment 119 were saved.

Clones from experiments 114, 115, 116, 117, 118, and 119 were selected based upon the following characteristics: brighter luminescence than Luc90-1B5, and increased retention of luminescence activity over time at pH 4.5. These select clones were shuffled together and were run on the robot as experiment 122. Eleven clones from this experiment were saved.

Three populations of random mutants were prepared from clone Luc122-4D5 and run on the robot as experiments 125, 126, and 127. Thirteen clones from experiment 125, four clones from experiment 126, and three clones from experiment 127 were shuffled together and run on the robot as experiment 128. For experiments 125, 126, 127 and 128 the screen for  $K_m$  was altered to select for clones that are more resistant to luciferin degradation products. The clones were also screened for retention of luminescence over time at pH 4.5.

20 Instead of screening for substrate utilization, a screen for resistance to inhibitor was conducted. In place of the 0.06X dilution of substrates, a 75:25 mix of D to L luciferin in 1X assay buffer was used and designated as "0.75X". In place of the 0.02X dilution of substrates, a 50:50 mix of D to L luciferin in 1X assay buffer and was designated as "0.5X". The 1X assay buffer in these experiments contained the following: 10  $\mu$ M ATP, 50 mM HEPES pH 7.8, 8 mM  $MgSO_4$ , and 0.1 mM EDTA. The 0.75X sample contained 75  $\mu$ M D-luciferin and 25  $\mu$ M L-luciferin. The 0.5X sample contained 50  $\mu$ M D-luciferin and 50  $\mu$ M L-luciferin. The 1X sample contained 250  $\mu$ M D-luciferin. A  $K_m$  regression was used as before and a  $K_m$  value was calculated. Normalized values of greater than 1 indicate more resistance to inhibitor. Clones from these experiments that were shown to have greater resistance to L-luciferin were also more resistant to luciferin degradation products.

To more easily measure resistance to inhibitor on the robotic system, a new variable "Q" was designated. The "Q" variable replaces the  $K_m$  variable used previously. The luminescence ratio is calculated the same as in the  $K_m$  measurement, then the natural log (ln) of each luminescence ratio is calculated (Y-axis). The X-axis is an arbitrary time that is entered by the user. The first time point is zero and the samples are measured with 1X assay buffer that contains 250  $\mu$ M D-luciferin. The next two time points have the same time value (i.e., 4 hours to simulate incubation of luciferin) and samples are measured with 1X assay buffer that contains a 50:50 mixture (as described above) of D-luciferin to L-luciferin. A linear regression correlating ln(lum ratio) to time is calculated. Q is calculated as the  $\ln(0.5)/\text{slope}$ . Normalized values of "Q" greater than 1 indicate more resistance to inhibitor. Experiments 133 and higher were run using this program.

Sixteen clones from experiment 128 were shuffled with clones from experiment 122 and run on the robot as experiment 133. Two samples, Luc133-1B2 and Luc133-0D11, were selected as templates for random mutagenesis and run on the robot as experiments 145 and 146, respectively. The clone that showed an increased retention of luminescence over time at pH 4.5 and the most resistance to inhibitor was clone Luc146-1H2. Moreover, at pH 4.5 and 48°C, Luc133-1B2 and Luc146-1H2 had increased thermostability relative to Luc90-1B5, and increased resistance to inhibitor (Figures 54-61). A comparison of the luminescence signal for Luc49-7C6, Luc78-0B10, Luc90-1B5, Luc133-1B2, and Luc146-1H2 is shown in Figure 59. A comparison of the thermostability at 50°C for clones for Luc49-7C6, Luc78-0B10, Luc90-1B5, Luc133-1B2, and Luc146-1H2 is shown in Figure 60. Figures 55-58 show the nucleotide sequence encoding and the inferred amino acid sequence of Luc133-1B2 and Luc146-1H2.

Materials and Methods

Assay to detect resistance to luciferase inhibitor

A 10 mM stock solution of luciferin is incubated at 50°C in 50 mM HEPES, pH 7.8, to accelerate the production of luciferin breakdown products. At different time points an aliquot is removed and then placed at -20°C. After incubation is complete, assay reagent (100  $\mu$ M Luciferin, 1  $\mu$ M ATP, 50 mM

HEPES, pH 7.8 and 8 mM MgSO<sub>4</sub>) is prepared with luciferin from each of the different time points and a diluted lysate is then assayed with each assay reagent.

The lysate is prepared as follows. Overnight cultures of clones to be tested are prepared in LB supplemented with 100 µg/ml AMP. The cultures are  
5 diluted 1:10 in M-9 minimal media supplemented with 1 mM IPTG, 100 µg/ml AMP and grown for 3 hours at 30°C. Forty-five µl of cells is mixed with 20 µl of Buffer A and frozen. The mixture is thawed, 175 µl of Buffer B added, and the resulting mixture diluted 1:10 in Buffer C. A regression of luminescence versus time of luciferin incubation is then calculated, and from this graph half-  
10 life is extrapolated. A longer half-life means that the mutant being tested is more resistant to luciferin breakdown products.

### EXAMPLE 6

#### Mutagenesis Strategy from LucPplYG to Clone Luc81-6G01

15 The luciferases from the luminous beetle, *Pyrophorus plagiophthalmus*, had been shown previously to generate different colors of luminescence (LucPpl). Analysis of these luciferases revealed that the different colors were caused by discreet amino acid substitutions to their protein sequences. This allowed the possibility to make a pair of genetic reporters capable of emitting a  
20 multiplexed luminescent signal, thus enabling quantitation of two biomolecular events simultaneously from within the same living system.

Amino acid substituted LucPpl were prepared which have the following properties:

#### Physical stability of the luciferases

25 Although the luminescence activity of LucPpl within colonies of *E. coli* appeared to be thermostable to above 60°C, in lysates these luciferases had relatively low stability. They were particularly unstable in the presence of Triton X-100 detergent. When lysates are prepared containing the commonly used firefly luciferase, the enzyme retains greater than 90% activity over 5 hours at  
30 room temperature. In contrast, the activity of the LucPpl luciferases would decrease several fold over the same period.

The thermostabilities of the LucPpl luciferases are also near the physiological temperature of mammalian cells. The green-emitting luciferase

(LucPplGR) and red-emitting luciferase (LucPplRD) have different thermostabilities which may cause differences in the behaviors as genetic reporters within cells. The influence of temperature should be greatest near the point of denaturation for the enzymes, where small changes in temperature will have the greatest effect on protein structure. In contrast, temperature will have much less affect on protein structure when it is much below the denaturation point. Thus, the differential effect on two enzymes having slightly different denaturation temperatures will be less at relatively lower temperatures. It might therefore be preferable to have the denaturation temperature of the reporter enzyme significantly above the growth temperature of mammalian cells.

#### Spectral overlap between the luciferases

Although a method was developed to quantify each luciferase in a mixture by using colored filters, the ability to discriminate between the luciferases is limited by their spectral overlap. This overlap reduces the ability to accurately measure both luciferases if their luminescence intensities differ by more than 10 fold. If the intensities differ by more than 50 fold, the luminescence signal of the dimmer luciferase is obscured by the other. Thus, it would be preferable to further separate the luminescence spectra of the two luciferases.

Many different mutations were identified which shifted the luminescence spectrum towards the red. But the limit for red luminescence appears to be about 620 nm. Further effort at shifting the spectrum of the red-emitting luciferase into still longer wavelengths might have some benefit. It was found that green-shifting mutations were rare, however, an extensive analysis was not conducted. Measurements from native luciferases show some examples of luminescence below 530 nm, about 15 nm less than the green-emitting prototype enzyme.

#### Differential physical and enzymological characteristics

Ideally the two luciferase reporters would be identical in all characteristics except for the color of luminescence. However, as noted above, the physical stability of the luciferases was not identical. It was also found that mutations resulting in red-shifted luminescence also caused an increase in the  $K_M$  for luciferin. Although some of these differences may be unavoidable, it is not clear whether the properties are fundamentally associated. For instance,

luciferases from different beetle species sometimes have significantly differing  $K_M$  even though their luminescence spectra are similar. It may be that much of the differences associated with development of the red-emitting luciferase are due to concomitant perturbations to the integrity of the enzyme structure, as the  
5 thermostability of a prototype of the red-emitting luciferase was increased without significantly altering the luminescence spectrum.

#### Stable luminescence signals

When firefly luciferase was first described as a genetic reporter, the luminescent signal was a relatively brief flash initiated upon injection of the  
10 reaction substrates. Subsequent development of the luminescent chemistry made the assay more convenient by enabling a stable signal for several minutes. Presently, such stabilized assays are standard for general laboratory applications. However, to allow high throughput screening in pharmaceutical research, the luminescence signal was further stabilized to extend for over an hour. This was  
15 necessary to allow sufficient time to assay several thousand samples in a batch. Although the luminescent signal of the new multiplexed luciferases was stable for minutes, they did not provide the extended signal stability needed for high throughput screening. It would be preferable if the signal stability could be further increased while optimizing other properties.

#### 20 Methods to Optimize Luciferase Performance

To prepare luciferases having certain performance, a method for *in vitro* evolution of enzyme function, as described above, was employed. Briefly described, the method is a recursive process of generating random mutations and screening for desirable properties. It was originally developed primarily to  
25 increase the thermostability of luciferases, although other enzymological characteristics are also subject to optimization by the screening criteria. A slightly different strategy was used to achieve the properties described above since two related luciferases needed to be optimized concomitantly.

Initially, a single prototype enzyme is subjected to *in vitro* evolution to  
30 optimize physical stability and the luminescence signal. In the process, the mutant libraries are also screened for any new mutations causing changes in color. Particular emphasis is placed on isolating green shifted mutants. After initial optimization of a common prototype, a green- and red-emitting form of



the enzymes is created, and these are further optimized separately to harmonize their physical and chemical properties. Particular attention is given to matching their physical stabilities and their substrate binding constants, especially for luciferin.

5           The choice for the initial prototype for optimization was the wild-type yellow-green-emitting luciferase isolated from the luminous beetles (*LucPpLYG*). Of the luciferases originally cloned from *P. plagiophthalmus*, this one produced the brightest luminescence when expressed in *E. coli*. Furthermore, there was concern that the lack of green-shifting mutation resulted because the prior mutagenesis studies were done using a luciferase that already  
10           had the greenest luminescence. It was possible that if additional green-shifting mutations existed, they might be more evident when screened in a red-shifted background. The mutagenesis was performed as follows:

Remove peroxisomal targeting sequence

15           The translocation signal at the C-terminus of the luciferases was removed. This was done using oligonucleotide-directed mutagenesis to convert the normal -KSKL to -XXX\* (where X represents any amino acid, and \* represents a termination codon). Several colonies yielding bright luminescence were selected and used as templates for the next stage of mutagenesis.

20           Removal of sensitive cysteines

          The luciferases from *P. plagiophthalmus* have 13 cysteines, which are potentially sensitive to oxidation. This is in contrast to the commonly used firefly luciferase, which has only 4 cysteines. To remove any cysteines that may limit enzyme stability, oligonucleotide-directed mutagenesis was used to  
25           randomize the cysteine codons. Three sets of oligonucleotides were used: non-conserved cysteines in regions of low sequence homology (positions 69, 114, 160, 194, 335, and 460), non-conserved cysteines in regions of higher sequence homology (positions 127, 213, 310, and 311), and highly conserved cysteines (positions 60, 80, and 388). The best clones from each of these screens were  
30           isolated, and a new mutant library made by sPCR and screened again. At the screening temperature of 29°C, the activity of the wild-type yellow-green-emitting luciferase decreased about 500-fold over 10 hours. The activity of the most stable mutant (Luc20-4C10) was more stable, decreasing only about 2 fold.

### First cycle of random mutagenesis

Using the procedure developed previously, three mutant libraries were generated using error-prone PCR and screened. The best mutants from these were recombined into a new library by sPCR and screened again. Finally, the  
5 best clones of this screen were recombined with the best clones from the previous oligonucleotide-directed mutagenesis by sPCR, and screened again. At 41°C, the activity of the best mutant from this process (Luc30-4B02) decreased 63 fold over 10 hours, whereas the activity of the parent mutant (Luc20-4C10) decreased greater than 100,000 fold.

### 10 Sequence analysis

Six of the best mutants from the last screen were isolated and sequenced. This revealed that the amino acids at 16 positions had been changed among the six clones. Thirteen positions had been changed in the preferred mutant, Luc30-4B02. Four of the changes were at the C-terminus in all the isolated mutants,  
15 where oligonucleotide mutagenesis had changed the wild-type sequence of –KSKL to –AGG\*. Only two of the cysteines had been changed by the previous oligonucleotide mutagenesis; one highly conserved cysteine at position 60 was changed to valine and one moderately conserved cysteine at position 127 was changed to threonine. The remaining amino acid changes were all due to point  
20 mutations in the DNA, consistent with error-prone PCR. Interestingly, three of these changed the amino acid into that found in the wild-type green-emitting luciferase (two in mutant Luc30-4B02). Four of the remaining changes brought the mutant sequences closer to the consensus amino acid among other cloned beetle luciferases (two in mutant Luc30-4B02) (Figure 19B). An additional 4  
25 codons were changed without affecting the amino acid sequence.

### Site-directed mutagenesis

To further explore the potential of the mutations identified in the sequenced mutants, additional mutagenesis experiments were performed using oligonucleotides. Eight of the codons mutated by the error-prone PCR were  
30 randomized or partially randomized using oligonucleotide-directed mutagenesis. Four of the remaining cysteine codons were randomized; two highly conserved cysteines (positions 80 and 388) and two cysteines in a region of sequence homology (positions 310 and 311). One leucine was mutated to a

leucine/proline; proline is the consensus amino acid among other beetle luciferases.

The mutagenesis was performed with four sets of oligonucleotides (Table 13), and the best clones from each set were selected. These were  
 5 recombined by sPCR together with the selected clones from the previous random mutagenesis and screened again. The activity of the best clone from this process (Luc47-7A11) decreased 2.3 fold at 42°C; the activity of the parent clone (Luc30-4B02) decreased greater than 2000 fold.

10

**Table 13**

Experiment	Mutations
Set A	$C_{80} \rightarrow X + K_{84} \rightarrow X + I_{91} \rightarrow (F, L, I, M, V, S, P, T, A)$
Set B	$I_{288} \rightarrow (F, L, I, M, V, S, P, T, A) \quad C_{310} \rightarrow X + C_{311} \rightarrow X$
15 Set C	$G_{351} \rightarrow (I, M, V, T, A, N, K, D, E, S, R, G) + L_{350} \rightarrow (L, P) + S_{356} \rightarrow P + L_{359} \rightarrow (F, L, I, M, V, S, P, T, A)$
Set D	$C_{388} \rightarrow X + V_{389} \rightarrow (NYN) \quad K_{457} \rightarrow X$

#### Second cycle of random mutagenesis

The random mutagenesis process using error-prone PCR was applied  
 20 again to the best clone from the oligonucleotide-directed mutagenesis (Luc47-7A11). Three libraries were again created and screened, and selected mutants were recombined by sPCR and screened again. Following recombination, the activity of the best mutant (Luc53-0G01) decreased 1.2 fold at 43°C. The parent clone (Luc47-7A11) decreased 150 fold. After recombining the best of these  
 25 new mutants with the best mutants from the previous oligonucleotide-directed mutagenesis, the activity of the new best mutant (Luc55-2E09) decreased 31 fold at 47°C, compared to 80 fold for the parent (Luc53-0G01).

#### Third cycle of random mutagenesis

The random mutagenesis process was repeated using the best clone from  
 30 the previous cycle of mutagenesis (Luc53-0G01). After recombining the selected mutants with the mutants from the second cycle of mutagenesis, the activity of the best clone (Luc81-6G01) decreased 100-fold at 47°C, compared with 750 fold for the parent (Luc53-0G01). The discrepancy in measured activity of Luc53-0G01 in this cycle of mutagenesis compared to the previous

cycle may be due to changes in the assay procedure and recalibration of the incubator temperature. It should be noted that the recorded thermostabilities from each stage of mutagenesis are calculated from robotic data using abbreviated assay procedures. The data are intended to indicate the relative stabilities of enzyme mutants when assayed in the same screen, rather than providing an accurate quantitation of thermostability.

#### Luminescence

Before making a final selection of the best clone from which to create the green- and red-emitting luciferases, further analysis was done on the best clones from the final screen. Three clones in particular were strong candidates as the final choice: Luc81-0B11, Luc81-5F01, and Luc81-6G01. The luminescence properties of these three mutant enzymes were compared among one another. They were also compared to the wild-type yellow-green-emitting luciferase to gauge the effect of the *in vitro* evolution process.

From colonies of *E. coli* expressing the luciferases, Luc81-5F01 and Luc81-6G01 produced luminescence most rapidly at room temperature upon addition of luciferin. The luminescence was more rapid and brighter than colonies expressing the wild-type green- and yellow-green-emitting luciferases. The luminescence from all the selected colonies appeared green-shifted compared to the yellow-green parent clone. When the colonies are heated to 65°C, the yellow-green clone loses most luminescence and the green clone becomes dimmer. Some of the mutant clones lose their luminescence at 65°C, but the three preferred clones remain bright above 70°C. No spectral changes upon heating the colonies were evident until above 70°C, where those clones still retaining activity began to red-shift slightly (sometime, the initial phases of enzyme denaturation are accompanied by a red shift in the luminescence). The luminescence characteristics of the three preferred mutants are quite similar.

The thermostability of the mutant luciferases in cell lysates was compared at room temperature (Figure 63). Dilute lysates were buffered at pH 7.5 and contained 1% Triton X-100; typical conditions for lysates of mammalian cells. The luminescence activity of all three mutant enzymes showed no decrease

over 20 hours, whereas the activity of the wild-type yellow-green-emitting luciferase decreased substantially.

For luminescence assays requiring only a few second, the wild-type yellow-green-emitting luciferase produces a very stable signal (the initial rise in the signal evident in the first 2 seconds is due to the response time of the luminometer, not the kinetics of the luminescence reaction) (Figure 64A). However, the signal intensity was reduced about 30% by the presence of 1% Triton X-100 in the lysate (diluted 1:5 with the addition of assay reagent). In contrast, the luminescence intensity of the mutant luciferases was unaffected by the presence of Triton X-100. Under these conditions, the most stable signal was produced by Luc81-6G01, although the signal intensity was somewhat brighter for Luc81-5F01. However, the data are not corrected for the efficiency of enzyme expression in *E. coli*. Thus, differences in luminescence intensity may not correlate to changes in enzyme specific activity, nor is the expression efficiency in *E. coli* necessarily relevant to expression in mammalian cells.

For batches of assays requiring more than an hour to process, the signal stability of the yellow-green-emitting luciferase is inadequate under the conditions tested. The luminescence intensity decreases several fold per hour (Figure 64B). Attempts to correct this by the *in vitro* evolution yielded mixed results. The signal stability of all three mutant enzymes was generally much improved over the parent yellow-green enzyme for three hours after substrate addition. However, this was accompanied by a greater initial decrease in luminescence during the first half-hour. This initial decrease would be more acceptable if it had occurred more rapidly, so that batch processing of samples would not be delayed by 30 minutes in waiting for the signal to stabilize. It may be possible to improve this kinetic behavior by adjusting the assay conditions.

From these results, the mutant Luc81-6G01 was chosen as the best clone from which to subsequently create the green- and red-emitting luciferases. The sequence of Luc81-6G01 (Figures 46-47) and Luc81-0B11 was determined and compared with the sequences of Luc30-4B02 from earlier in the *in vitro* evolution process, and the wild-type yellow-green-emitting luciferase used as the initial parent clone (Figure 19B). Relative to Luc30-4B02, the Luc81-6G01 mutant acquired new mutations in 9 codons, of which 8 caused changes in the

amino acid sequence. Four of these 8 amino acid changes were probably acquired through recombination with clones generated prior to isolation of Luc30-4B02. Two are identical to mutations found in the other clones sequenced along with Luc30-4B02, and two are reversions to the wild-type parent sequence. The remaining four are novel in the sequence of Luc81-6G01. Two of the novel mutations change the amino acid to the consensus amino acid among other cloned luciferases.

Interestingly, in either the sequences of Luc81-6G01 or Luc81-0B11, there is no evidence that the prior oligonucleotide-directed mutagenesis had any beneficial effect. No novel nucleotide sequences appear at any of the targeted codons. The improved enzyme performance following the oligonucleotide-directed mutagenesis apparently was due to recombination of previously acquired mutations. All of the novel amino acid changes in Luc81-6G01 and Luc81-0B11 are at sites not targeted by the oligonucleotides and are due to single-base modifications of the codons, consistent with error-prone PCR. Even though the novel mutations in Luc81-6G01 were not found in the earlier sequence data, it is not certain when they were generated in the process. Most likely they were produced in the second and third cycles of random mutagenesis; however, they may have been present among other selected mutants prior to Luc30-4B02. Relative to the initial yellow-green-emitting luciferase, the Luc81-6G01 mutant has acquired 17 amino acid changes and 3 codon mutations not affecting the amino acid sequence.

The observation that the onset of luminescence within colonies of *E. coli* is faster for the new mutants, and that the luminescence is brighter at higher temperatures, is probably not due to differences in protein expression. Immunoblot analysis of cell expressing the different luciferases showed no significant differences in the amount of polypeptide present. As noted above, the greater light intensity at higher temperatures is due to the increased thermostability of the mutant luciferases. The apparent  $K_M$ 's for ATP and luciferin have also changed during the course of the *in vitro* evolution (Table 14). To estimate the  $K_M$  values, the mutant luciferases were partially purified from lysates of *E. coli* by differential precipitation using ammonium sulfate (40-

65% saturation fraction). The results show that the  $K_M$ 's for both ATP and luciferase are more than 10-fold lower.

When luciferin is added to an *E. coli* colony expressing luciferase, the intracellular concentration of luciferin slowly increases as it diffuses across the cell membrane. Thus, the intracellular concentration of luciferin reaches saturation sooner for those luciferases having the lowest  $K_M$ 's. Hence, the mutant luciferases appear brighter sooner than the wild-type parent clone. This also explains why the luminescence of the red-emitting prototype clone appears in *E. coli* colonies much more slowly than the green-emitting luciferase.

Analysis of  $K_M$  shows that the mutations causing the red luminescence also substantially increase the  $K_M$  for luciferin.

Table 14

Luciferase	$K_M$ for ATP ( $\mu$ M)	$K_M$ for luciferin ( $\mu$ M)
YG w.t.	140	21
Luc30-4B02	12	7.8
Luc81-6G01	8.0	1.9

From the analysis of luminescence signal *in vitro*, the luminescence from the mutant luciferases might be expected to fade more quickly than the wild-type luciferase during the first 30 minutes. Following this, the luminescence should be most stable in the mutants. However, this has not been noticed in the colonies of *E. coli*, and it may be that the kinetics of luminescence are different within cells compared to diluted enzyme in buffer.

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All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification, this invention has been described in relation to certain preferred embodiments thereof, and many details  
20 have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details herein may be varied considerably without departing from the basic principles of the invention.



**WHAT IS CLAIMED IS:**

1. A second luciferase that has enhanced resistance to an inhibitor of the luciferase relative to a first reference beetle luciferase.
2. The second luciferase of claim 1 which retains at least 50% more activity in the presence of the inhibitor relative to the reference beetle luciferase.
3. The second luciferase of claim 1 which retains at least 100% more activity in the presence of the inhibitor relative to the reference beetle luciferase.
4. The second luciferase of claim 1 which comprises a plurality of amino acid substitutions relative to the reference beetle luciferase.
5. The second luciferase of claim 4 wherein the reference luciferase is native beetle luciferase.
6. The second luciferase of claim 5 wherein the reference beetle luciferase is *LucPpl*.
7. The second luciferase of claim 5 wherein the reference beetle luciferase is *LucPpe2*.
8. The second luciferase of claim 4 wherein the substitutions are to a consensus amino acid.
9. A luciferase which comprises SEQ ID NO:14, SEQ ID NO:19, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:47, or an enzymologically active portion thereof.
10. An isolated and purified nucleic acid molecule comprising a nucleic acid segment encoding the luciferase of claim 9 or the complement thereof.

11. The isolated and purified nucleic acid molecule of claim 10 comprising a nucleic acid segment comprising SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:42, SEQ ID NO:43 or SEQ ID NO:46.
12. An isolated and purified nucleic acid molecule comprising a nucleic acid segment encoding the second luciferase of claim 1 or the complement thereof.
13. A vector containing the nucleic acid molecule of claim 10.
14. A vector containing the nucleic acid molecule of claim 12.
15. A host cell, the genome of which is augmented with the nucleic acid molecule of claim 10.
16. A host cell, the genome of which is augmented with the nucleic acid molecule of claim 12.
17. A solid substrate comprising the second luciferase of claim 1.
18. A solid substrate comprising the luciferase of claim 9.
19. A fusion protein comprising the second luciferase of claim 1.
20. A fusion protein comprising the luciferase of claim 9.
21. An isolated and purified nucleic acid molecule comprising a nucleic acid segment encoding the fusion protein of claim 19.
22. An isolated and purified nucleic acid molecule comprising a nucleic acid segment encoding the fusion protein of claim 20.

23. A method of using a luciferase, comprising: linking an agent with the second luciferase of claim 1 so as to yield a labeled agent.
24. The use of the second luciferase of claim 1 for detecting ATP, for labeling a molecule, as a genetic reporter, for immobilization onto a solid surface, to produce hybrid proteins, for high temperature reactions, or for creating luminescent solutions.
25. A method of using a luciferase, comprising: linking an agent with the luciferase of claim 9 so as to yield a labeled agent.
26. The use of the luciferase of claim 9 for detecting ATP, for labeling a molecule, as a genetic reporter, for immobilization onto a solid surface, to produce hybrid proteins, for high temperature reactions, or for creating luminescent solutions.
27. A method of using a vector encoding a luciferase, comprising:
  - a) introducing the vector of claim 13 into a host cell; and
  - b) detecting or determining the presence of luciferase in the host cell.
28. A method of using a vector encoding a luciferase, comprising:
  - a) introducing the vector of claim 14 into a host cell; and
  - b) detecting or determining the presence of luciferase in the host cell.
29. A kit comprising: a container comprising the second luciferase of claim 1.
30. The kit of claim 29 wherein the container comprises an aqueous mixture comprising the luciferase.
31. The kit of claim 29 wherein the container comprises lyophilized luciferase.

32. The kit of claim 29 further comprising a container comprising luciferin.
33. The kit of claim 29 wherein the container further comprises luciferin.
34. The kit of claim 31 wherein the container further comprises lyophilized luciferin.
35. The kit of claim 34 wherein the container which comprises luciferin comprises lyophilized luciferin.
36. The kit of claim 29 further comprising packaging material enclosing, separately packaged, the container and instruction means directing the user to correlate the luciferase activity in a sample suspected of having ATP with the level of ATP in the sample.
37. The kit of claim 29 further comprising packaging material enclosing, separately packaged, the container and instruction means directing the user to correlate the luciferase activity in a sample suspected of having an infectious agent that produces ATP with the level or presence of the agent in the sample.
38. A kit comprising: a container comprising the luciferase of claim 9.
39. The kit of claim 38 wherein the container comprises an aqueous mixture comprising the luciferase.
40. The kit of claim 38 wherein the container comprises lyophilized luciferase.
41. The kit of claim 38 further comprising a container comprising luciferin.
42. The kit of claim 38 wherein the container further comprises luciferin.

43. The kit of claim 40 wherein the container further comprises lyophilized luciferin.
44. The kit of claim 41 wherein the container which comprises luciferin comprises lyophilized luciferin.
45. The kit of claim 38 further comprising packaging material enclosing, separately packaged, the container and instruction means directing the user to correlate the luciferase activity in a sample suspected of having ATP with the level of ATP in the sample.
46. The kit of claim 38 further comprising packaging material enclosing, separately packaged, the container and instruction means directing the user to correlate the luciferase activity in a sample suspected of having an infectious agent that produces ATP with the level or presence of the agent in the sample.
47. A method to prepare an enzyme which is not a beetle luciferase and which has enhanced enzymological properties, comprising:
- a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is not a luciferase and which has at least one enhanced enzymological property from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme which is not a luciferase, wherein the first isolated polynucleotide sequence is subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has at least one enhanced enzymological property relative to the enzyme encoded by the first isolated polynucleotide sequence;
  - b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences, wherein the selected isolated polynucleotide sequence is subjected to oligonucleotide mediated mutagenesis with a plurality of oligonucleotides each

comprising at least one codon that encodes a consensus amino acid which is not present in the first polynucleotide sequence; and  
c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme which is not a luciferase having at least one enhanced enzymological property and comprising a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence.

48. The method of claim 47 further comprising isolating the further polynucleotide sequence.
49. The method of claim 47 wherein in step b) a mixture of the selected isolated polynucleotide sequences of step a) are mutated.
50. The method of claim 47 wherein the property is specific activity, enzyme activity, catalytic turnover,  $K_m$  or substrate utilization.
51. The method of claim 47 wherein the enzyme is DNA polymerase or RNA polymerase.
52. The method of claim 47 where the enzyme is detectable in crude cellular lysates or cells.
53. The method of claim 52 wherein the enzyme is chloramphenicol acetyltransferase, beta-glucuronidase or beta-galactosidase.
54. The method of claim 47 wherein the first polynucleotide sequence is subjected to recombination mutagenesis.
55. The method of claim 47 wherein the first polynucleotide sequence is subjected to point mutagenesis.

56. The method of claim 47 wherein the selection is an automated multi-parameter process.
57. A polynucleotide sequence which is obtained by the method of claim 47.
58. A method to prepare an enzyme that is resistant to an inhibitor, comprising:
- a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is resistant to an inhibitor from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has increased resistance to an inhibitor relative to the enzyme encoded by the first isolated polynucleotide sequence;
  - b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences; and
  - c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme that is resistant to an inhibitor and comprises a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence.
59. The method of claim 58 further comprising isolating the further polynucleotide sequence.
60. The method of claim 58 wherein in step b) a mixture of the selected isolated polynucleotide sequences of step a) are mutated.
61. The method of claim 58 wherein the further polynucleotide sequence encodes an enzyme that has increased thermostability relative to the first polynucleotide sequence.

62. The method of claim 58 wherein the enzyme is DNA polymerase or RNA polymerase.
63. The method of claim 58 wherein the enzyme is resistant to inhibition by a substrate analog of the enzyme.
64. The method of claim 58 wherein the mutating employs oligonucleotides having at least one codon encoding a consensus amino acid.
65. The method of claim 58 wherein the enzyme is a luciferase.
66. The method of claim 65 wherein the luciferase is a beetle luciferase.
67. The method of claim 66 wherein the first polynucleotide sequence encodes *LucPpe2*.
68. The method of claim 66 wherein the first polynucleotide sequence encodes *LucPpl*.
69. The method of claim 58 wherein a plurality of amino acid substitutions are to a consensus amino acid.
70. The method of claim 58 wherein the first polynucleotide sequence is subjected to recombination mutagenesis.
71. The method of claim 58 wherein the first polynucleotide sequence is subjected to point mutagenesis.
72. The method of claim 58 wherein the selection is an automated multi-parameter process.
73. The method of claim 65 wherein the luciferase has increased luminescence intensity, increased signal stability or decreased  $K_m$ .



- 74. A polynucleotide sequence which is obtained by the method of claim 58.
- 75. An enzyme which is encoded by the polynucleotide sequence of claim 57.
- 76. An enzyme which is encoded by the polynucleotide sequence of claim 76.

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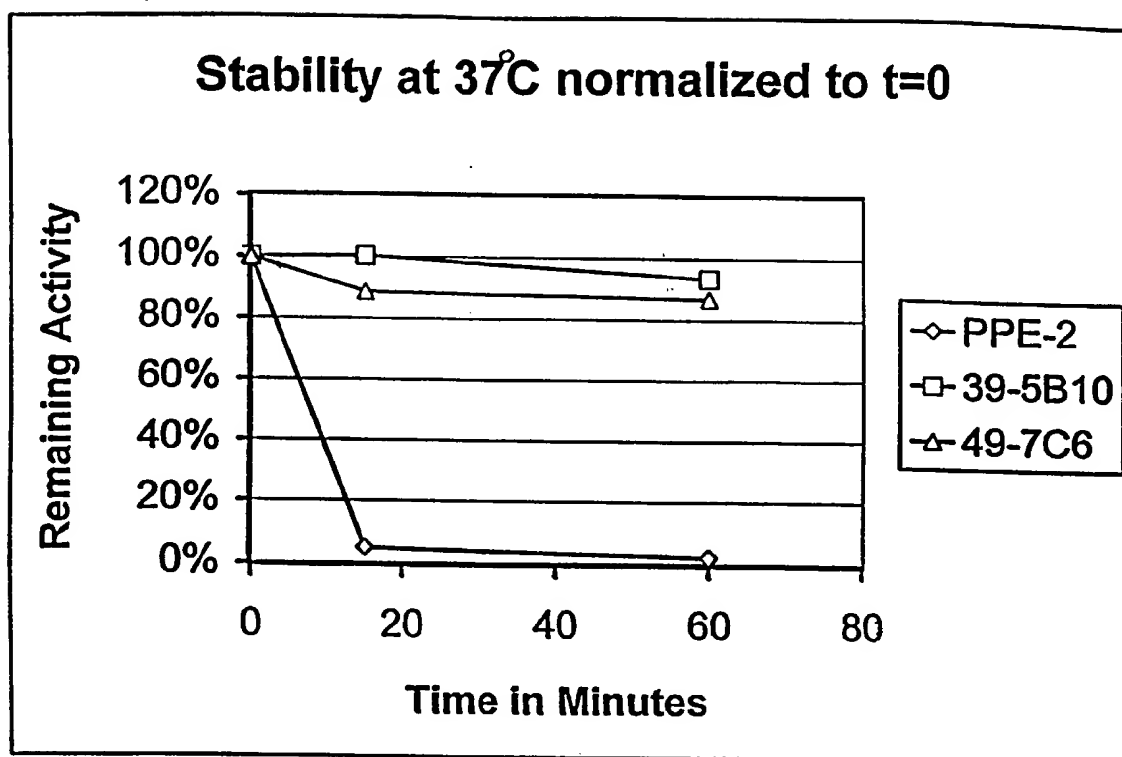


FIG. 1

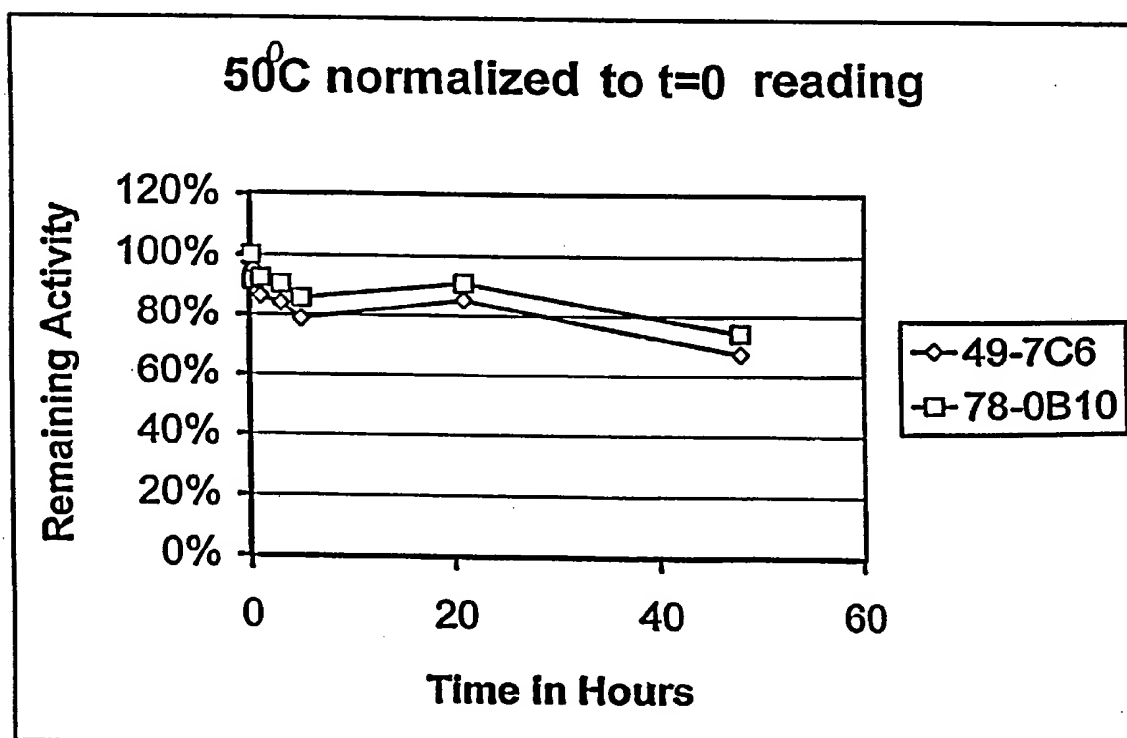


FIG. 2

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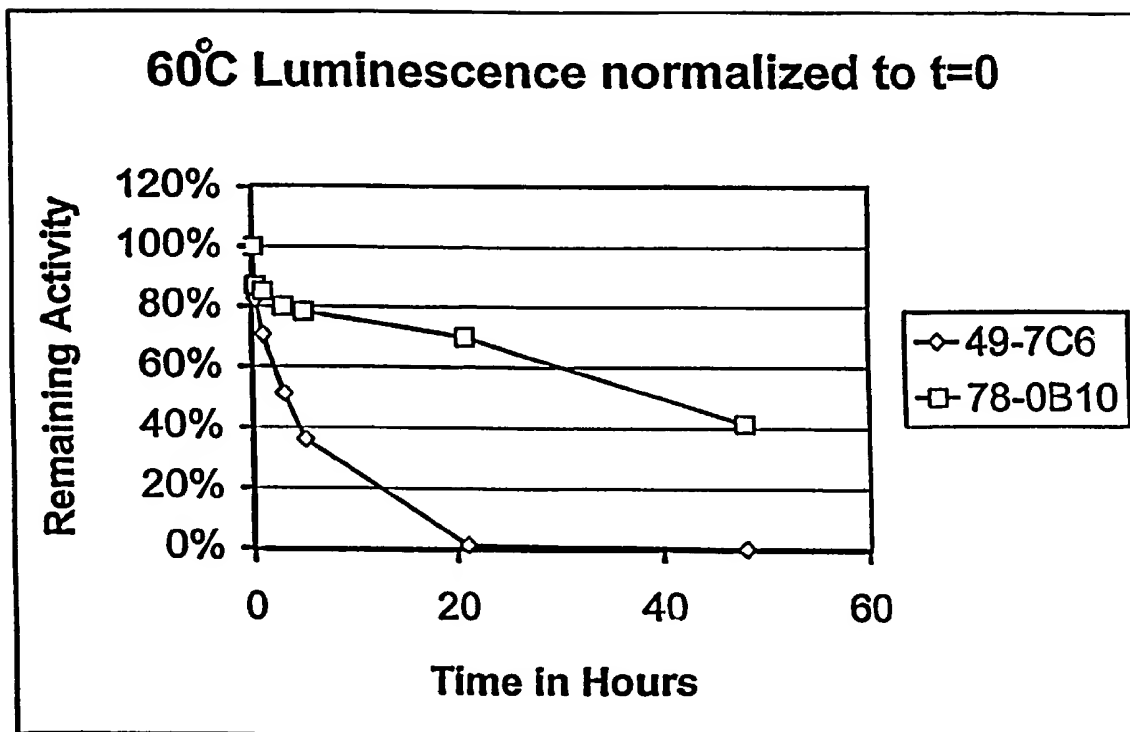


FIG. 3

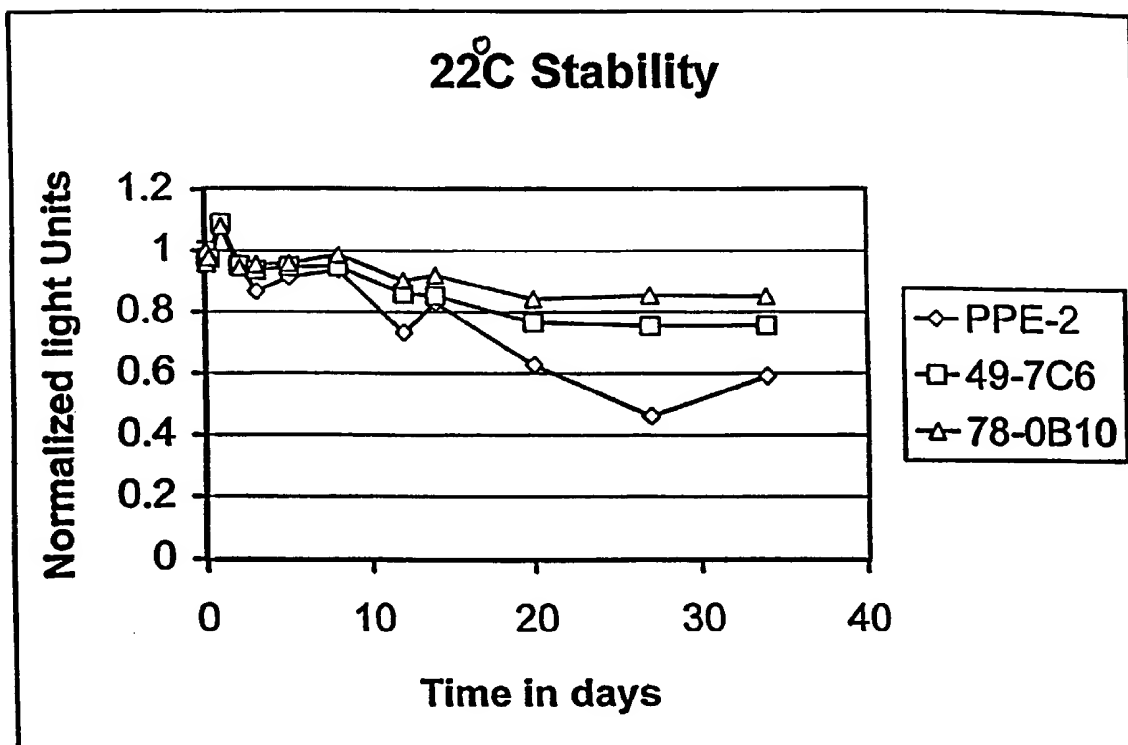


FIG. 4

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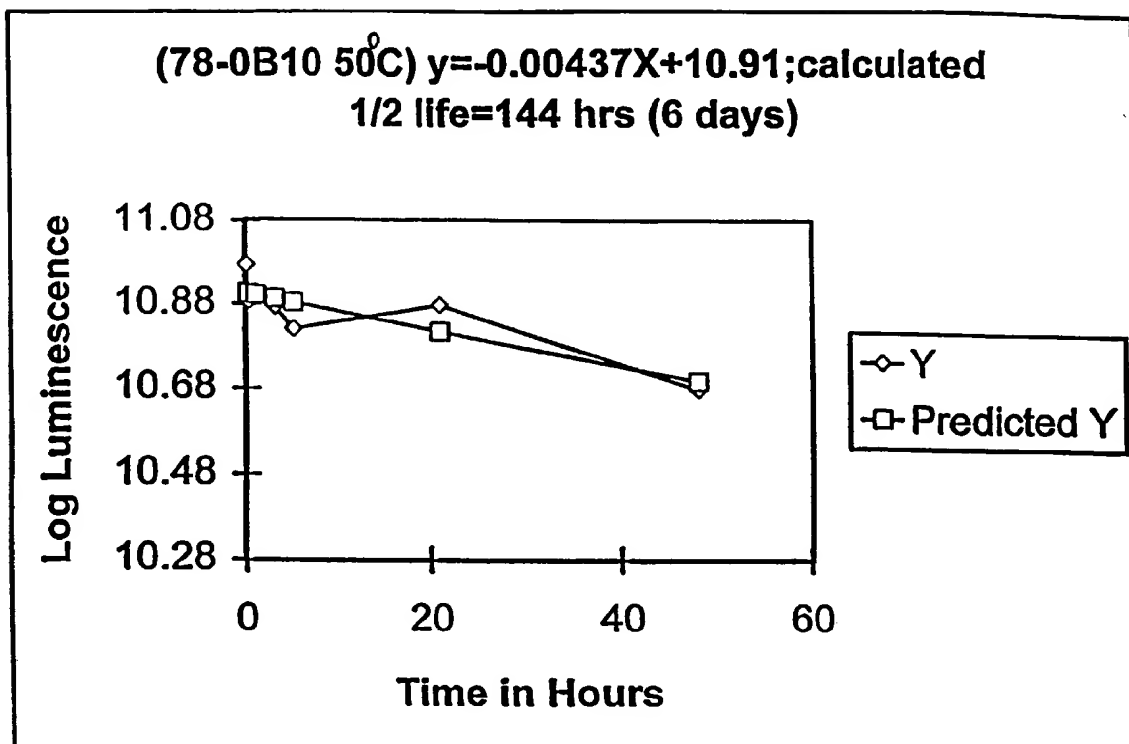


FIG. 5

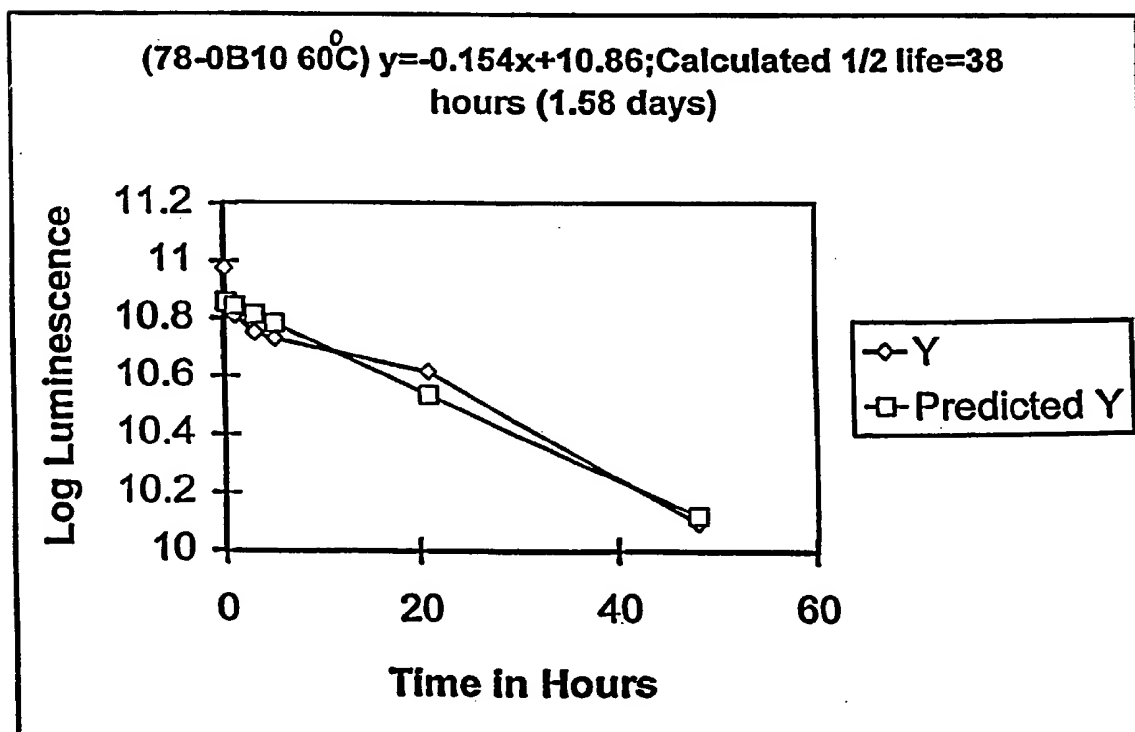


FIG. 6

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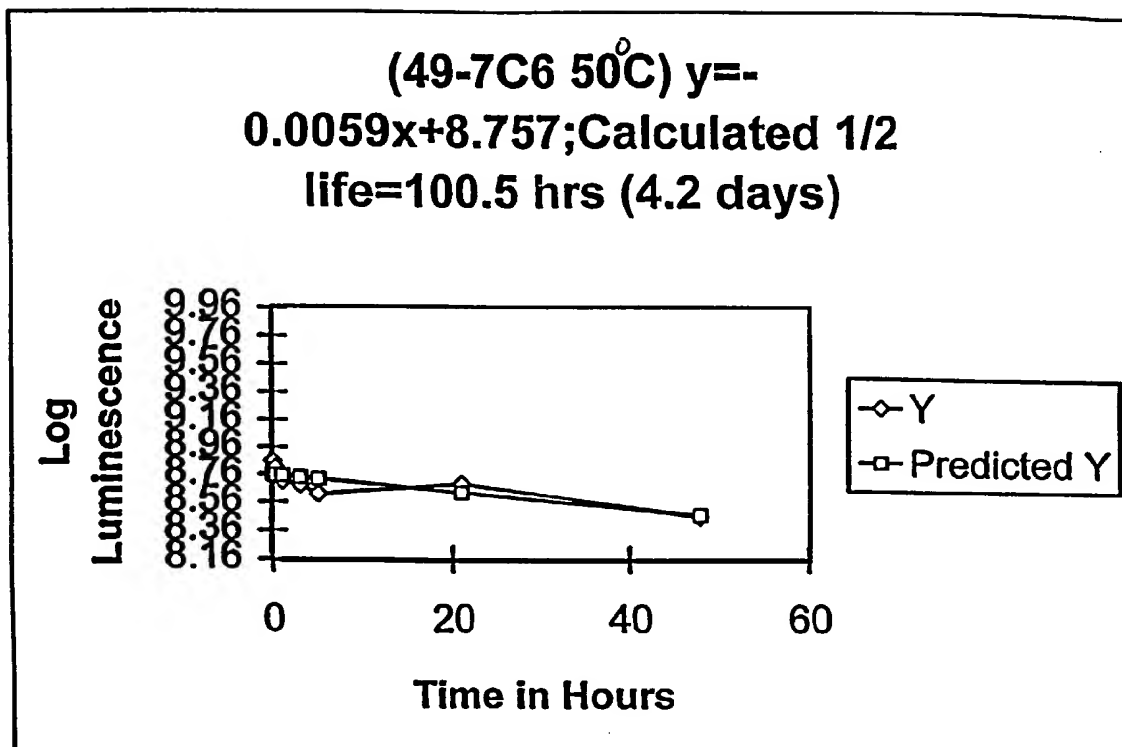


FIG. 7

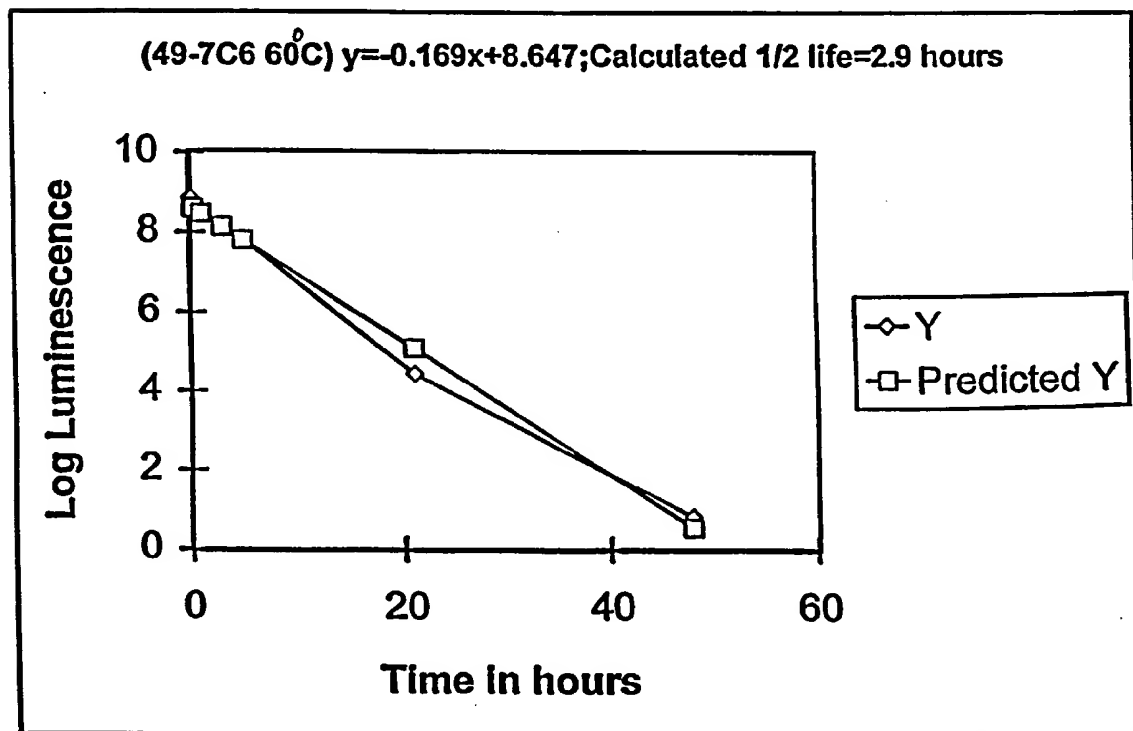


FIG. 8

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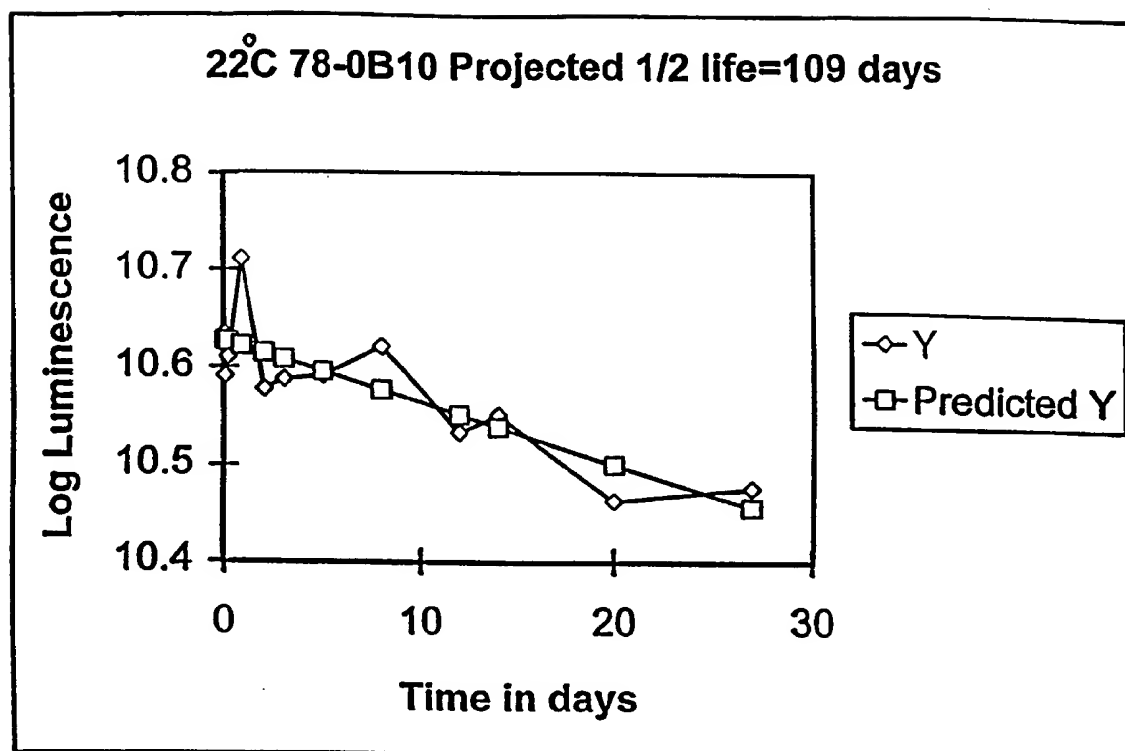


FIG. 9

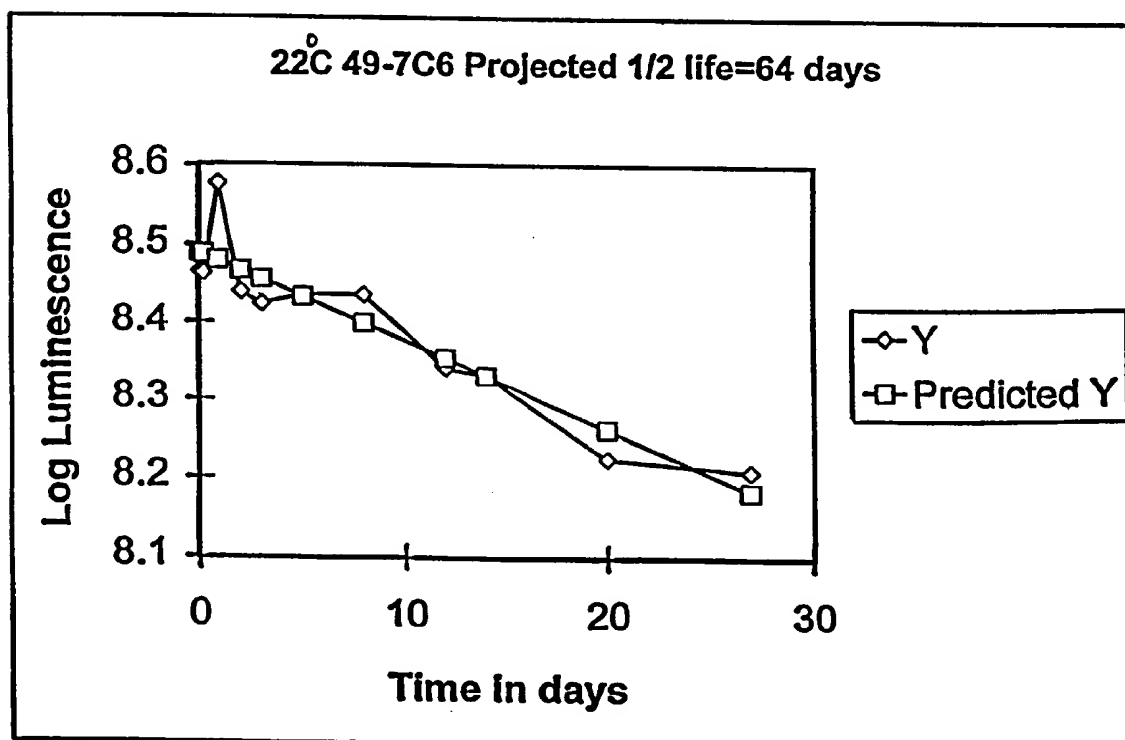


FIG. 10

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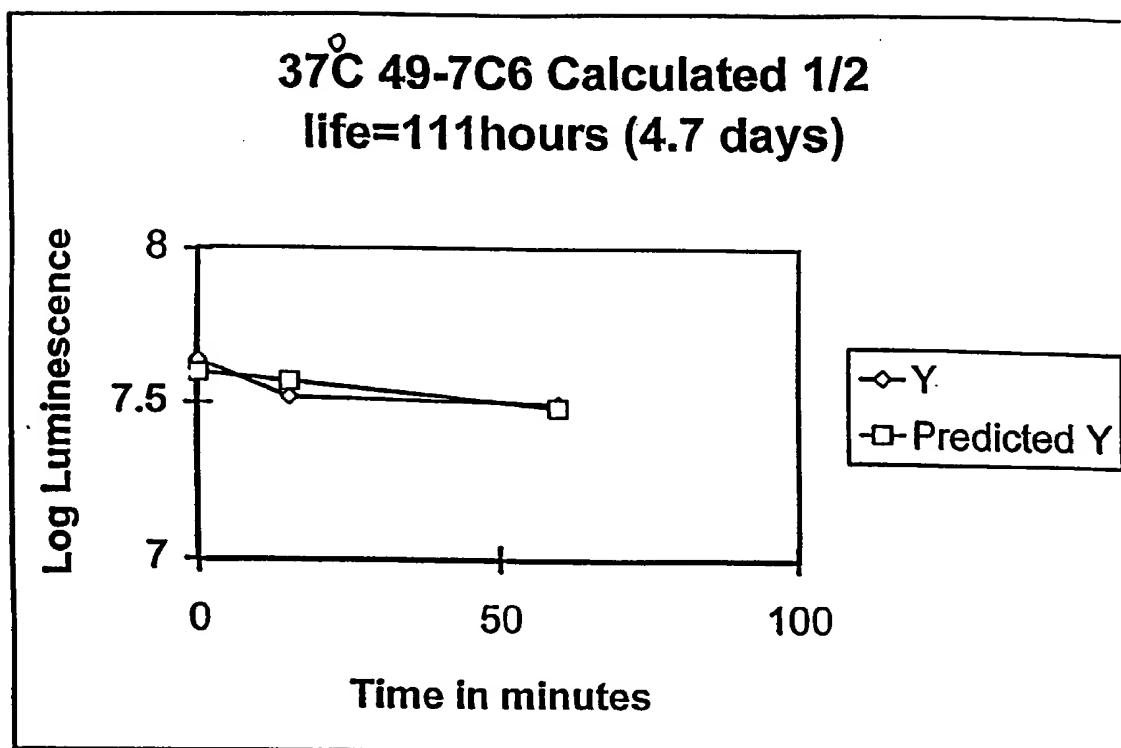


FIG. 11

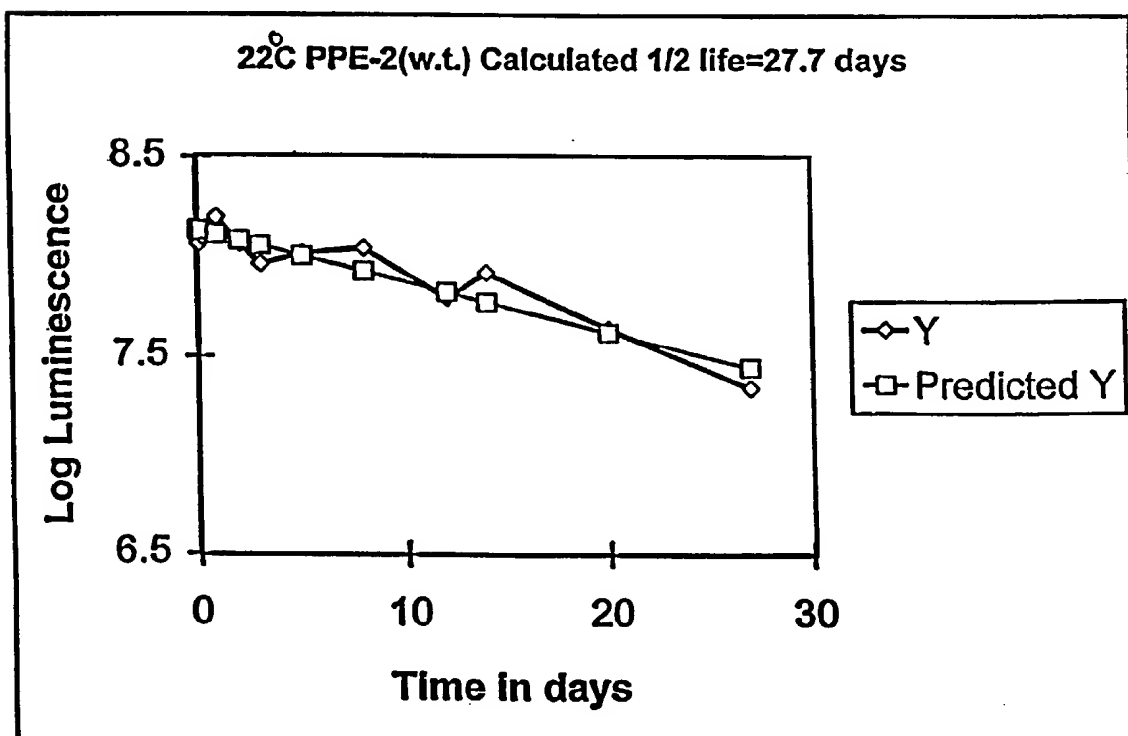


FIG. 12

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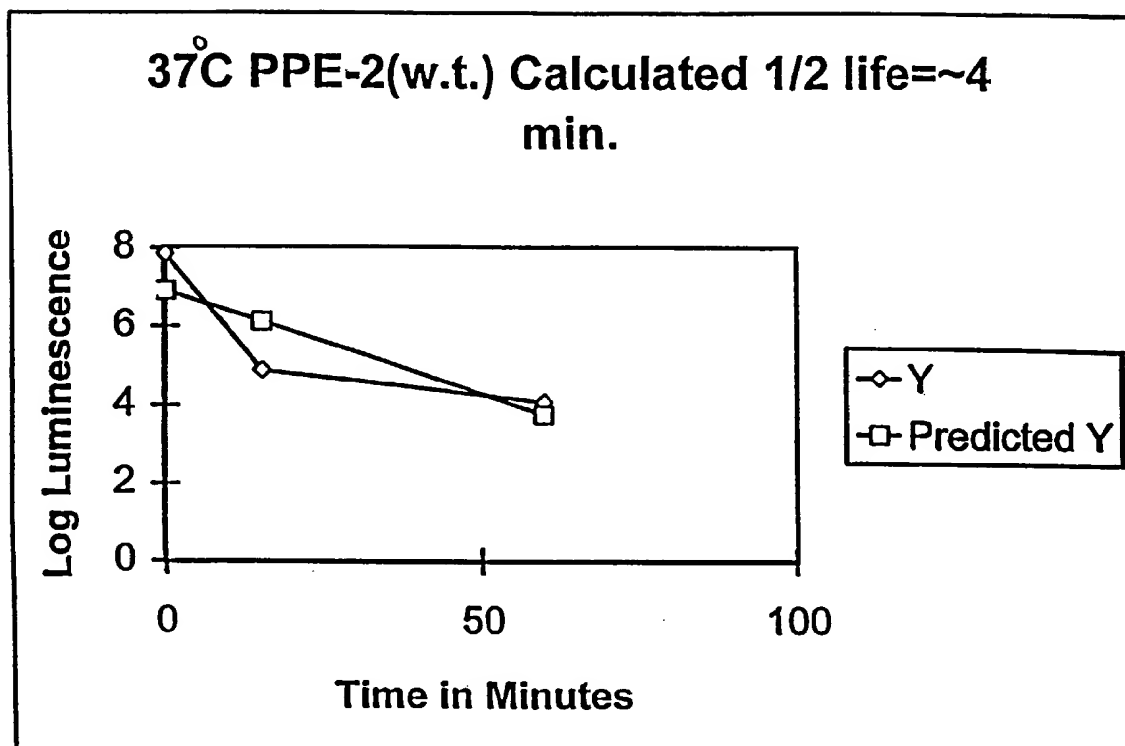


FIG. 13



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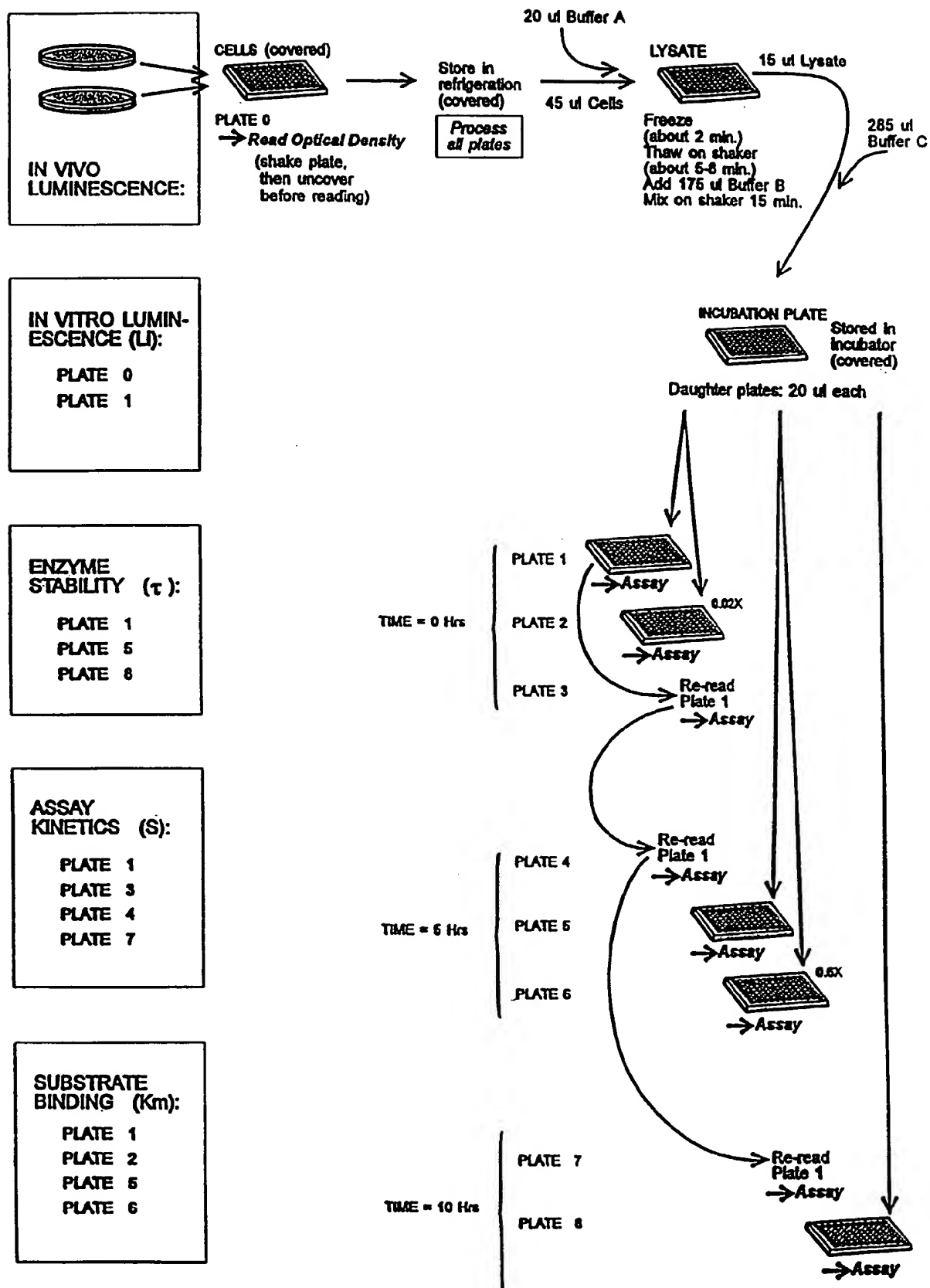
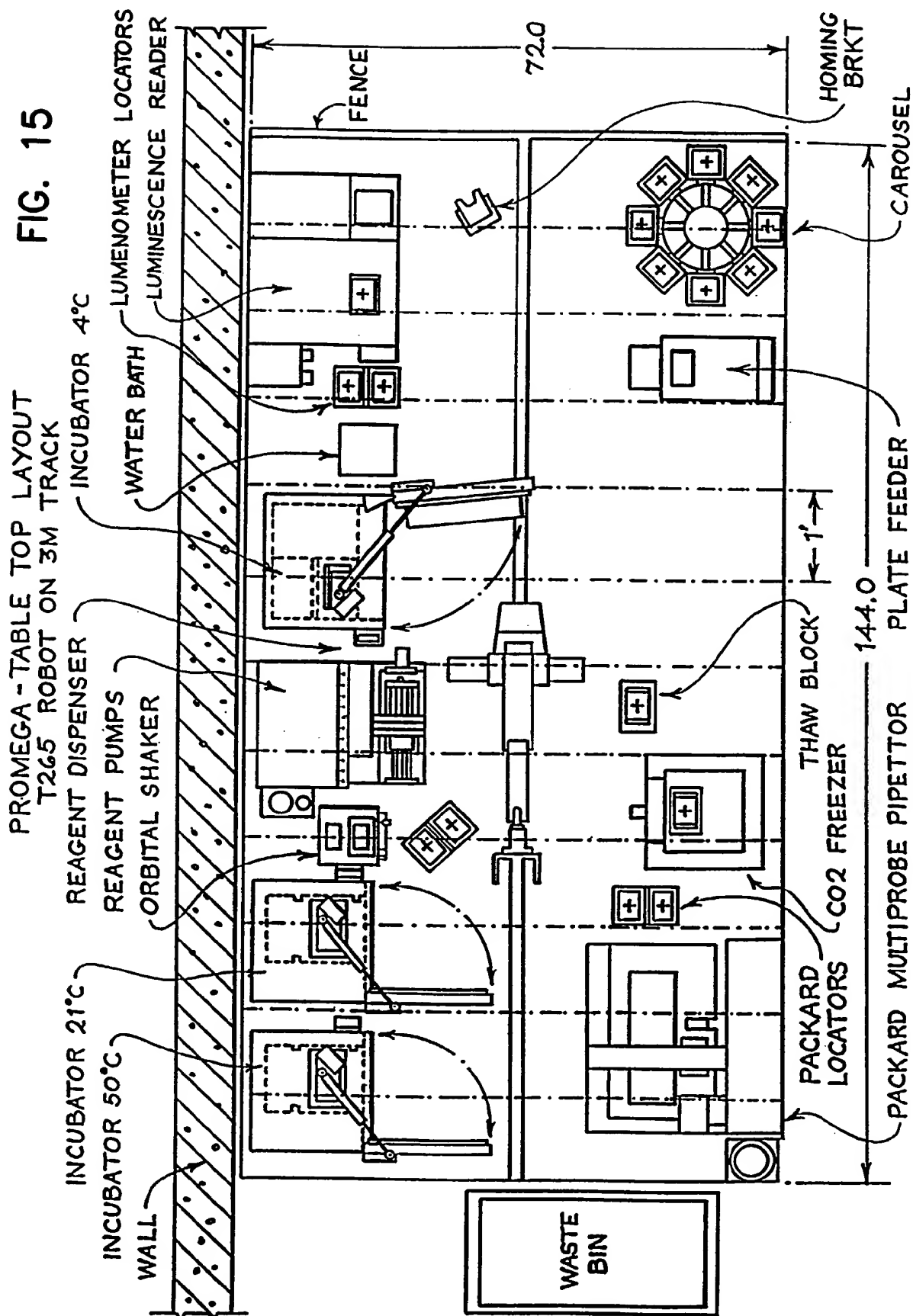


FIG. 14

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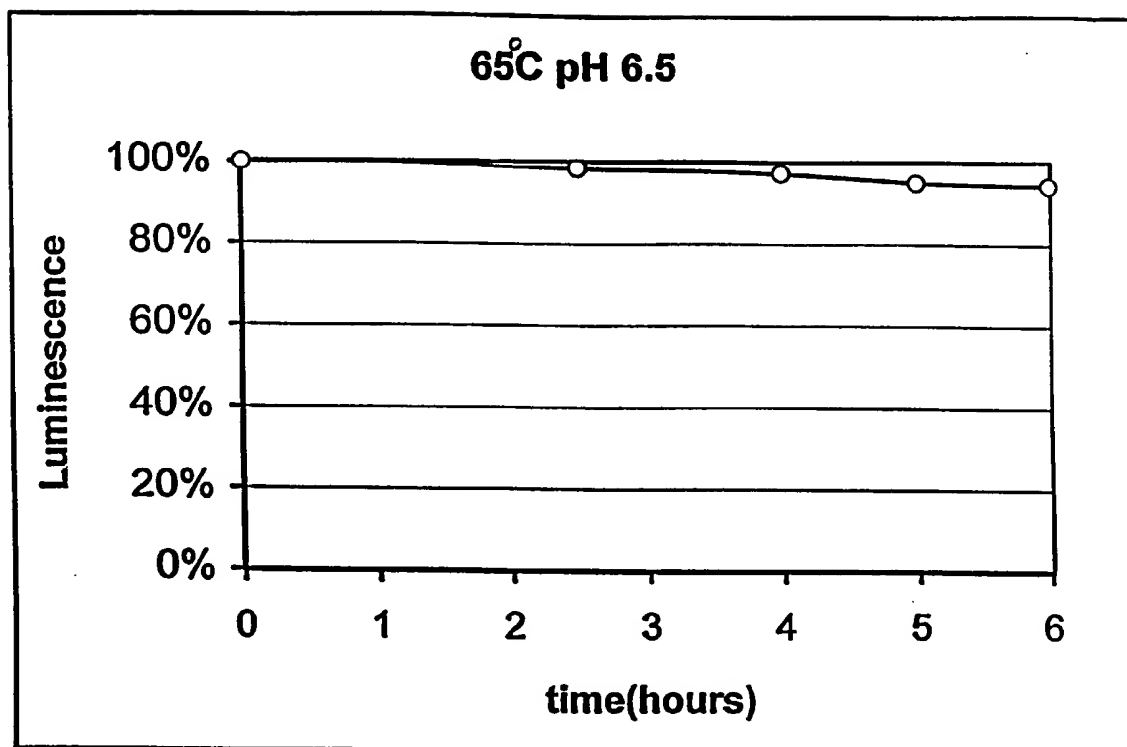


FIG. 16A

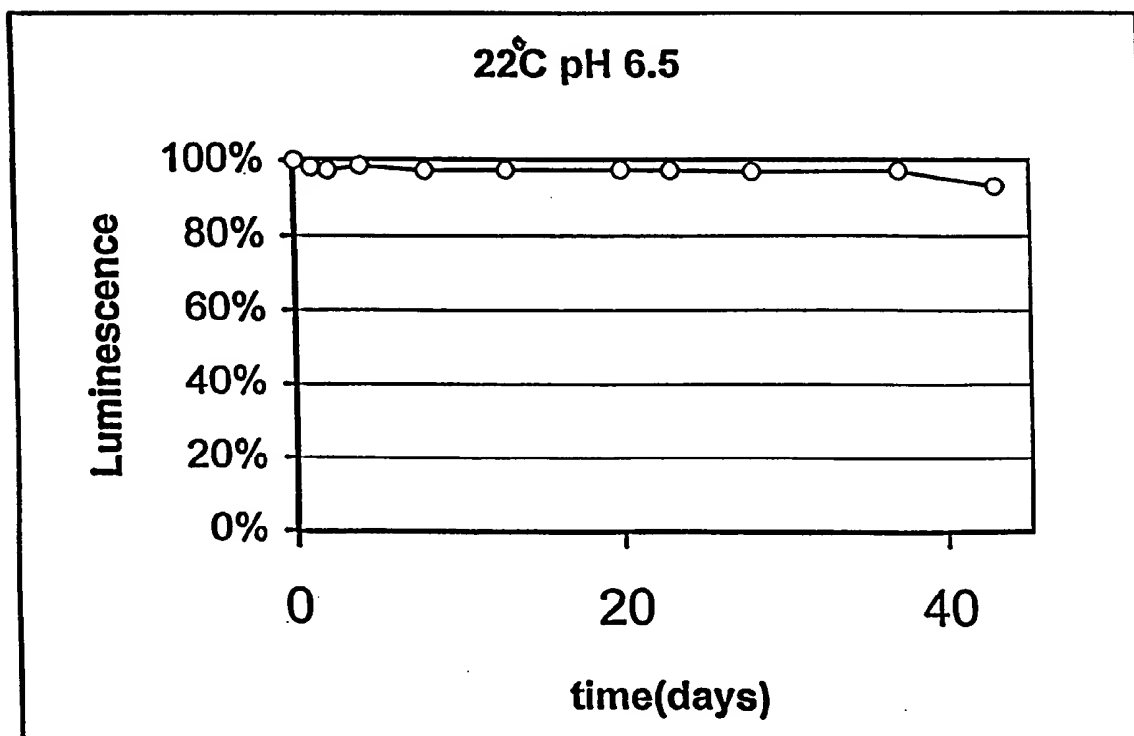


FIG. 16B

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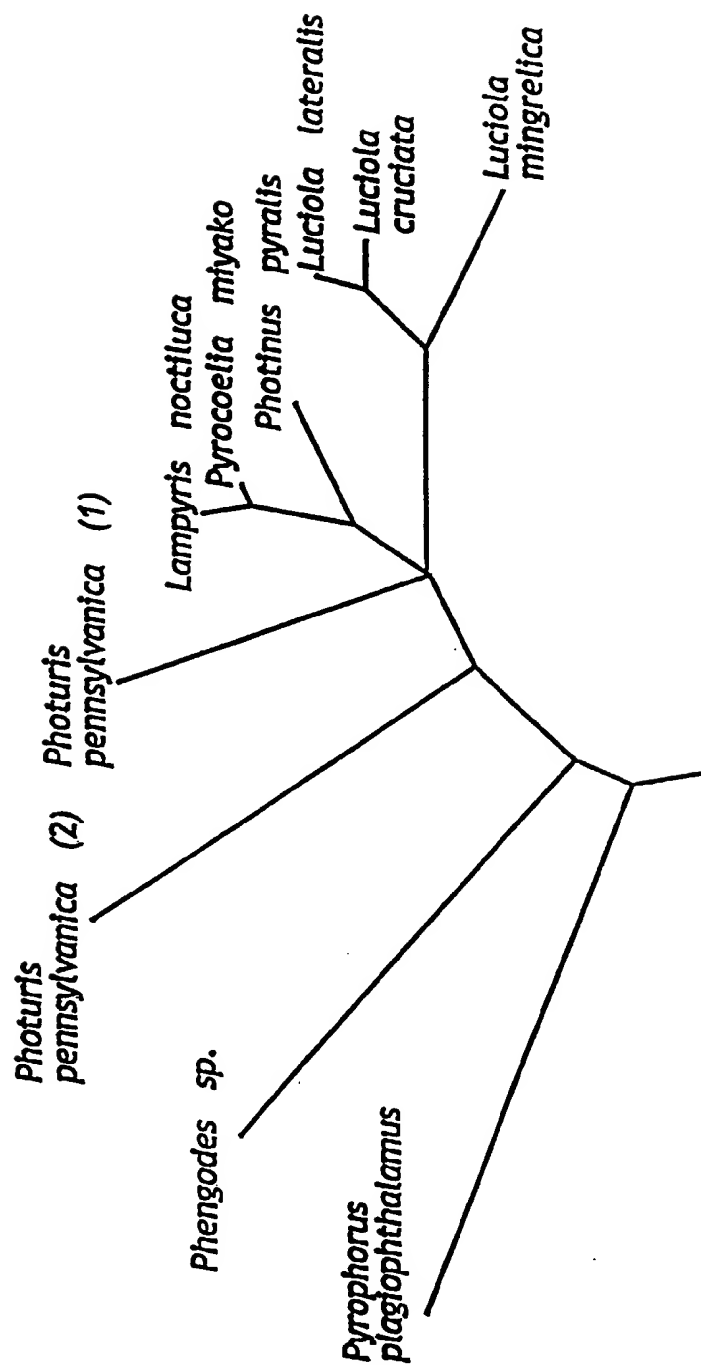


FIG. 17



FIG. 18A

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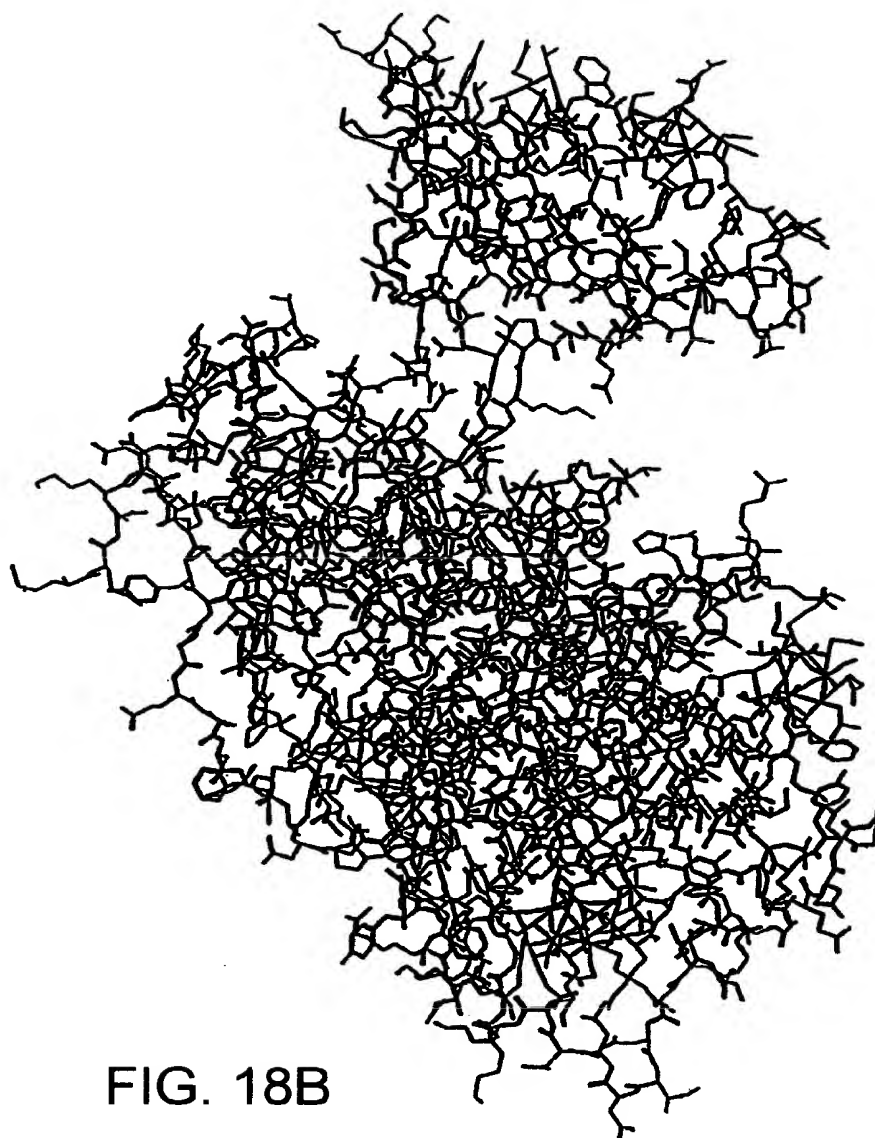


FIG. 18B

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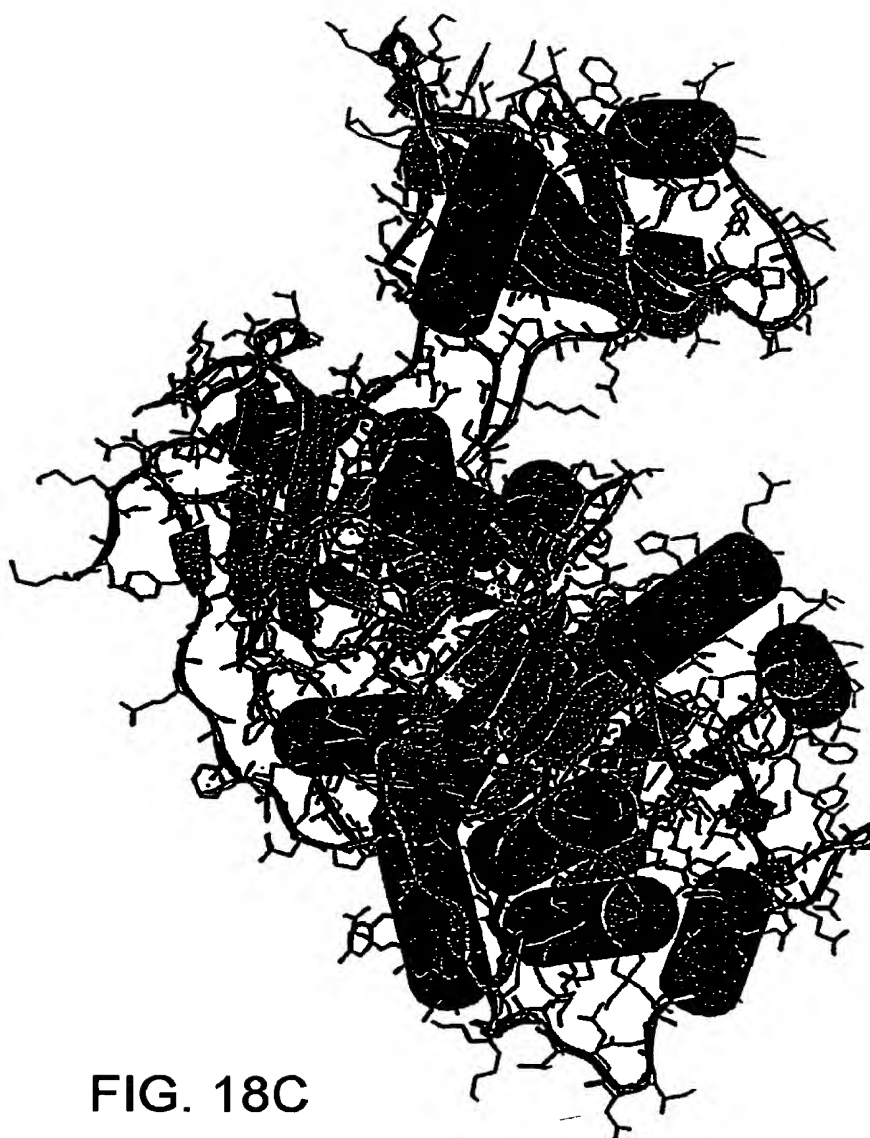


FIG. 18C

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	1				50
Lcr	MENMENDE.N	IVVGPKPFYP	IEEGSAGTQL	RKYMERYAKL	.GAIIFTNAV
Lla	MENMENDE.N	IVYGPEPFYP	IEEGSAGAQL	RKYMDRYAKL	.GAIIFTNAL
Lmi	ME.MEKEE.N	VVYGPLPFYP	IEEGSAGIQL	HKYMHQYAKL	.GAIAFSNAL
Pmi	...MEDDSKH	IMHGHRHSIL	WEDGTAGEQL	HKAMKRYAQV	PGTIAFTDAH
Ppy	...MED.AKN	IKKGPAFPYP	LEDGTAGEQL	HKAMKRYALV	PGTIAFTDAH
Lno	...MED.AKN	IMHGPAFPYP	LEDGTAGEQL	HKAMKRYAQV	PGTIAFTDAH
Ppe1	...MSI.ENN	ILIGPPPYYP	LEEGTAGEQL	HRAISRYAAV	PGTLAYTDVH
Phg	MIKME..EEH	VMPGAMPRL	LFEGTAGQQL	HRALYKHSYF	PE..AIVDSH
GR	...MMKREKN	VVYGPEPLHP	LEDLTAGEML	FRALRKHSHL	PQ..ALVDVY
YG	...MMKREKN	VIYGPEPLHP	LEDLTAGEML	FRALRKHSHL	PQ..ALVDVF
Ppe2	...MED..KN	ILYGPEPFYP	LADGTAGEQM	FYALSRYADI	SGCIALTNAH
49-7C6	A				
78-0B10	A			D	
90-1B5	A		E	D	P
133-1B2	A		E	D	P
146-1H2	A		E	D	A P
Cons	---M-----	---G-----	---AG---	-----	---A-----
	51				100
Lcr	TGVDYSYAAY	LEKSCCLGKA	LQNYGLVVDG	RIALCSENCE	EFFIPVLAGL
Lla	TGVDYTYAAY	LEKSCCLGEA	LKNYGLVVDG	RIALCSENCE	EFFIPVLAGL
Lmi	TGVDISYQAY	FDITCRLAEA	MKNFGMKPEE	HIALCSENCE	EFFIPVLAGL
Pmi	AEVNITYSEY	FEMSCRLAET	MKRYGLGLQH	HIAVCSETSL	QFFMPVCGAL
Ppy	IEVNITYAAY	FEMSVRLAEA	MKRYGLNTNH	RIVVCSENSL	QFFMPVLGAL
Lno	AEVNITYSEY	FEMACRLAET	MKRYGLGLQH	HIAVCSENSL	QFFMPVCGAL
Ppe1	TELEVITYKEF	LDVTCRLAEA	MKNYGLGLQH	TISVCSENCV	QFFMPICAAAL
Phg	THEIISYAKI	LDMSCRLAVS	FQKYGLTQNN	IIGICSENNL	NFFNPVIAAF
GR	GEEWISYKEF	FETTCLLAQS	LHNCGYKMND	VVSICAENNK	RFFVPIIAAW
YG	GDESLSYKEF	FEATCLLAQS	LHNCGYKMND	VVSICAENNK	RFFVPIIAAW
Ppe2	TKENVLYEFP	LKLSCRLAES	FKKYGLKQND	TIAVCSENGL	QFFPLPIASL
49-7C6					I
78-0B10					V
90-1B5					V
133-1B2				S	V
146-1H2				S	V
Cons	-----Y---	-----L---	-----G-----	-----C-E---	-FF-P-----
	101				150
Lcr	FIGVGVAPT	EIYTLRELVE	SLGISKPTIV	FSSKKGLDKV	ITVQKTVTI
Lla	FIGVGVAPT	EIYTLRELVE	SLGISKPTIV	FSSKKGLDKV	ITVQKTVATI
Lmi	YIGVAVAPT	EIYTLRELVE	SLGIAQPTIV	FSSRKGLPKV	LEVQKTVTI
Pmi	FIGVGVAPT	DIYNERELYN	SLFISQPTIV	FCSKRALQKI	LGVQKKLPVI
Ppy	FIGVAVAPT	DIYNERELYN	SMNISQPTIV	FVSKKGLQKI	LNQVQKKLPVI
Lno	FIGVGVAPT	DIYNERELYN	SLSISQPTIV	SCSKRALQKI	LGVQKKLPVI
Ppe1	YGVVATAPT	DIYNERELYN	SLSISQPTIV	FTSRNSLQKI	LGVQSRPLPI
Phg	YLGITVATVN	DTYTDRELS	TLNITKQML	FCSKQSLPIV	MKTMKIMPVY
GR	YIGMIVAPVN	EGYIPDELCK	VMGISRPQLV	FCTKNILNKV	LEVQSRDFI
YG	YIGMIVAPVN	ESYIPDELCK	VMGISRPQIV	FCTKNILNKV	LEVQSRDFI
Ppe2	YLGIIAAPVS	DKYIERELIH	SLGIVKPRII	FCSKNTFQKV	LNQVSKLKYV
49-7C6					
78-0B10					S
90-1B5	V	N		V	SI
133-1B2	V	N		V	SI
146-1H2	V	N		V	SI
Cons	--G---A---	--Y---EL---	---I---P---	-----	-----

FIG. 19A



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	151				200
Lcr	KTIVILDSKV	DYRGYQCLDT	FIKRNTPPGF	QASSFKTVEV	.DRKEQVALI
Lla	KTIVILDSKV	DYRGYQSM DN	FIKRNTPQGF	K3SSFKTVEV	.NRKEQVALI
Lmi	KTIVILDSKV	NFGGHDCMET	FIKKHVELGF	QPSSFVPIDV	KNRKQHVALI
Pmi	QKIVILDSRE	DYMGKQSMYS	FIESHLPPGF	NEYDYIPDSF	.DRETATALI
Ppy	QKIIIMDSKT	DYQGFQSMYT	FVTSHLPPGF	NEYDFVPESF	.DRDKTIALI
Lno	QKIVILDSRE	DYMGKQSMYS	FIESHLPPGF	NEYDYIPDSF	.DRETATALI
Ppe1	KKIIILDGKK	DYLGYSQMQS	FMKEHVPANF	NVSAFKPLSF	.DLDR.VACI
Phg	QKLLIIDSMQ	DIGGIECVHS	FVSRYTDEHF	DPLKFVPLDF	.DPREQVALI
GR	KRIIILDAVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHY	.DPVEQVA AI
YG	KRIIILDTVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHY	.DPVEQVA AI
Ppe2	ETIIILD LNE	DLGGYQCLNN	FISQNSDINL	DVKKFKPNSF	.NRDDQVALV
49-7C6				Y	
78-0B10			S	Y	
90-1B5			S	Y	I
133-1B2	D		S	Y	I
146-1H2			S	Y	SI
Cons	---I-D---	---G-----	F-----	-----	-----A--
	201				250
Lcr	MNSSGSTGLP	KGVLTHENT	VTRFSHARDP	IYGNQVSPGT	AVLTVVPFHH
Lla	MNSSGSTGLP	KGVLTHENA	VTRFSHARDP	IYGNQVSPGT	AILTVVPFHH
Lmi	MNSSGSTGLP	KGVRITHEGA	VTRFSHARDP	IYGNQVSPGT	AILTVVPFHH
Pmi	MNSSGSTGLP	KGVDLTHMNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH
Ppy	MNSSGSTGLP	KGVALPHRTA	CVRFSHARDP	IFGNQIIPDT	AILSVVPFHH
Lno	MNSSGSTGLP	KGVELTHQNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH
Ppe1	MNSSGSTGLP	KGVPISHRNT	IYRFSHCRDP	VFGNQIIPDT	TILCAVPFHH
Phg	MTSSGTTGLP	KGVM LTHRNI	CVRFVHSRDP	LFGTRFIPET	SILSLVPFHH
GR	LCSSGTTGLP	KGVMQTHRNV	CVRLIHALDP	RVGTQLIPGV	TVLVYLPFFH
YG	LCSSGTTGLP	KGVMQTHQNI	CVRLIHALDP	RAGTQLIPGV	TVLVYLPFFH
Ppe2	MFSSGTTGVS	KGVM LTHKNI	VARFSHCKDP	TFGNAINPTT	AILTVIPFHH
49-7C6			LA		
78-0B10	P		LA		
90-1B5	LP		LA		
133-1B2	LP		IA	S	
146-1H2	LP		IA	S	
Cons	--SSG-TG--	KGV---H---	--R--H--DP	--G----P--	--L---PF-H
	251				300
Lcr	GFGMFTTLGY	LICGFRVVML	TKFDEETFLK	TLQDYKCTSV	ILVPTLFAIL
Lla	GFGMFTTLGY	LTCGFRIVML	TKFDEETFLK	TLQDYKCQSV	ILVPTLFAIL
Lmi	GFGMFTTLGY	FACGYRVVML	TKFDEELFLR	TLQDYKCTSV	ILVPTLFAIL
Pmi	VFGMFTTLGY	LTCGFRIVLM	YRFEELFLR	SLQDYKIQSA	LLVPTLFSFF
Ppy	GFGMFTTLGY	LICGFRVVML	YRFEELFLR	SLQDYKIQSA	LLVPTLFSFF
Lno	GFGMFTTLGY	LTCGFRIVLM	YRFEELFLR	SLQDYKIQSA	LLVPTLFSFF
Ppe1	AFGTFTNLGY	LICGFHVLM	YRFNEHLFLQ	TLQDYKCQSA	LLVPTVLAFI
Phg	AFGMFTT LSY	FIVGLKIVMM	KRFDGELFLK	TIQNYKIPTI	VIAPPVMVFL
GR	AFGFSINLGY	FMVGLRVIML	RRFDQEAFLK	AIQDYEVRSV	INVPAIILFL
YG	AFGFSINLGY	FMVGLRVIML	RRFDQEAFLK	AIQDYEVRSV	INVPAIILFL
Ppe2	GFGMTTTLGY	FTCGFRVALM	HTFEEKLFLQ	SLQDYKVEST	LLVPTLMAFF
49-7C6	M	V			L
78-0B10	M	V			L
90-1B5	M	V			L
133-1B2	M	V			L
146-1H2	M	V			L
Cons	-F-----L-Y	---G-----	--F---FL-	--Q-Y-----	---P-----

FIG. 19A (Continued)

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	301				350
Lcr	NKSELLNKYD	LSNLVEIASG	GAPLSKEVGE	AVARRFNLPG	VRQGYGLTET
Lla	NRSELLDKYD	LSNLVEIASG	GAPLSKEIGE	AVARRFNLPG	VRQGYGLTET
Lmi	NKSELIDKFD	LSNLTEIASG	GAPLAKEVGE	AVARRFNLPG	VRQGYGLTET
Pmi	AKSTLVDKYD	LSNLHEIASG	GAPLAKEVGE	AVAKRFKLPG	IRQGYGLTET
Ppy	AKSTLIDKYD	LSNLHEIASG	GAPLSKEVGE	AVAKRFHLPG	IRQGYGLTET
Lno	AKSTLVDKYD	LSNLHEIASG	GAPLAKEVGE	AVAKRFKLPG	IRQGYGLTET
Ppe1	AKNPLVDKYD	LSNLHEIASG	GAPLSKEISE	IAAKRFKLPG	IRQGYGLTET
Phg	AKSHLVDKYD	LSSIKEIATG	GAPLGPALAN	AVAKRLKLG	IIQGYGLTET
GR	SKSPLVDKYD	LSSLRELCCG	AAPLAKEVAE	IAVKRLNLPG	IRCGPGLTES
YG	SKSPLVDKYD	LSSLRELCCG	AAPLAKEVAE	VAVKRLNLPG	IRCGPGLTES
Ppe2	AKSALVEKYD	LSHLKEIASG	GAPLSKEIGE	MVKKRPFKLN	VRQGYGLTET
49-7C6					
78-0B10					
90-1B5					
133-1B2					
146-1H2					
Cons	----L--K-D	LS---E---G	-APL-----	----R--L--	---G-GLTE-
	351				400
Lcr	TSAILIITPEG	DDKPGASGKV	VPLFKAKVID	LDTKKSLGPN	RRGEVCVKGP
Lla	TSAILIITPEG	DDKPGASGKV	VPLFKAKVID	LDTKKTLGPN	RRGEVCVKGP
Lmi	TSAFIITPEG	DDKPGASGKV	VPLFKVKVID	LDTKKTLGVN	RRGEICVKGP
Pmi	TSAILIITPEG	DDKPGACGKV	VPFFAKIVD	LDTGKTLGVN	QRGELCVKGP
Ppy	TSAILITPEG	DDKPGAVGKV	VPFFFAKVVD	LDTGKTLGVN	QRGELCVRGP
Lno	TSAILIITPEG	DDKPGACGKV	VPFFSAKIVD	LDTGKTLGVN	QRGELCVKGP
Ppe1	TCAIVITABG	EFKLGAVGKV	VPFYSLKVLD	LNTGKKLGPN	ERGEICFKGP
Phg	CCAVALITPHN	KIKTGSTGQV	LPYVTAKIVD	TKTGKNLGPN	QTGELCFKSD
GR	TSANIHSIRD	EFKSGSLGRV	TPLMAAKIAD	RETGKALGPN	QVGELCIKGP
YG	TSANIHSIRD	EFKSGSLGRV	TPLMAAKIAD	RETGKALGPN	QVGELCVKGP
Ppe2	TSAVLITPDT	DVRPGSTGKI	VPFHAVKVVD	PTTGKILGPN	ETGELYFKGD
49-7C6	NN				P
78-0B10	KG	A			P A
90-1B5	KG	AK			P P
133-1B2	KG	AK			P P
146-1H2	KG	AK	L		P P
Cons	--A-----	----G--G--	-P-----K--D	--T-K-LG-N	--GE-----
	401				450
Lcr	MLMKGYNPN	EATKELIDEE	GWLHTGDIGY	YDEEKHFFIV	DRLKSLIKYK
Lla	MLMKGVDNP	EATREIIDE	GWLHTGDIGY	YDEEKHFFIV	DRLKSLIKYK
Lmi	SLMLGYSNPN	EATRETIDEE	GWLHTGDIGY	YDEDEHFFIV	DRLKSLIKYK
Pmi	MIMKGYVNP	EATNALIDKD	GWLHSGDIAY	YDKDGHFFIV	DRLKSLIKYK
Ppy	MIMSGYVNP	EATNALIDKD	GWLHSGDIAY	WDEDEHFFIV	DRLKSLIKYK
Lno	MIMKGYVNP	EATSALIDKD	GWLHSGDIAY	YDKDGHFFIV	DRLKSLIKYK
Ppe1	MIMKGYLNP	EATRELIDEE	GWIHSGDIGY	FDEDGHVYIV	DRLKSLIKYK
Phg	IIMKGYQNE	EETRLVIDKD	GWLHSGDIGY	YDTDGNFHYV	DRLKELIKYK
GR	MVSKGYVNP	EATKEAIDDD	GWLHSGDFGY	YDEDEHFFIV	DRYKELIKYK
YG	MVSKGYVNP	EATKEAIDDD	GWLHSGDFGY	YDEDEHFFIV	DRYKELIKYK
Ppe2	MIMKSYNNE	EATKAIINKD	GWLRSGDIAY	YDNDGHFYIV	DRLKSLIKYK
49-7C6	G				
78-0B10	G		DN		
90-1B5	G		DN		
133-1B2	G		DN		
146-1H2	G		DN		
Cons	-----Y--N-	E-T---I---	GW---GD--Y	-D-----V	DR-K-LIKYK

FIG. 19A (Continued)

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451 500

Lcr GYQVPPAELE SVLLQHPSIF DAGVAGVPDP VAGELPGAVV VLESGKNMTE  
 Lla GYQVPPAELE SVLLQHPSIF DAGVAGVPDP IAGELPGAVV VLEKGSMTTE  
 Lmi GYQVPPAELE SVLLQHPSIF DAGVAGVPDP DAGELPGAVV VMEKGSMTTE  
 Pmi GYQVPPAELE SILLQHPSIF DAGVAGIPDP DAGELPAAVV VLEEGKMTTE  
 Ppy GYQVAPAELE SILLQHPSIF DAGVAGLPDD DAGELPAAVV VLEHGKMTTE  
 Lno GYQVPPAELE SILLQHPSIF DAGVAGIPDP DAGELPAAVV VLEEGKMTTE  
 Ppe1 GYQVPPAELE ALLLQHPSIF DAGVAGVPDE VAGDLPGAVV VLEKGSITE  
 Phg AYQVAPAELE ALLLQHPYIA DAGVTGIPDE EAGELPAACV VLEPGKMTTE  
 GR GSQVAPAELE EILLKNPCIR DVAVVGIPDL EAGELPSAFV VIQPGKEITA  
 YG GSQVAPAELE EILLKNPCIR DVAVVGIPDL EAGELPSAFV VKQPGKEITA  
 Ppe2 GYQVAPAEIE GILLQHPYIV DAGVTGIPDE AAGELPAAGV VVQTGKYLNE

49-7C6  
 78-0B10  
 90-1B5  
 133-1B2  
 146-1H2

Cons --QV-PAE-E --LL--P-I- D--V-G-PD- -AG-LP-A-V V---GK----

501 550

Lcr KEVMDYVASQ VSNKRLRGG VRFVDEVPKG LTGKIDGRA. IREILKKPV.  
 Lla KEVMDYVASQ VSNKRLRGG VRFVDEVPKG LTGKIDGKA. IREILKKPV.  
 Lmi KEIVDYVNSQ VVNHKRLRGG VRFVDEVPKG LTGKIDAKV. IREILKKPQ.  
 Pmi QEVMDYVAGQ VTASKRLRGG VKFVDEVPKG LTGKIDSRK. IREILTMGQK  
 Ppy KEIVDYVASQ VTTAKKL RGG VVFVDEVPKG LTGKIDARK. IREILIKAKK  
 Lno QEVMDYVAGQ VTASKRLRGG VKFVDEVPKG LTGKIDGRK. IREILMMGKK  
 Ppe1 KEIQDYVAGQ VTSSKKLRGG VEFVKEVPKG FTGKIDTRK. IKEILIKAQK  
 Phg KEVMDYLAER VTPTKRLRGG VLFVNNIPKG ATGKLVRTE. LRRLLTQRA.  
 GR KEVYDYLAEV VSHTKYL RGG VRFVDSIPRN VTGKITRKEI. LKQILLEKS..  
 YG KEVYDYLAEV VSHTKYL RGG VRFVDSIPRN VTGKITRKEI. LKQILLEKS..  
 Ppe2 QIVQNFVSSQ VSTAKWL RGG VKFLDEIPKG STGKIDRKV. LRQMFKEH..  
 49-7C6  
 78-0B10 D  
 90-1B5 DY A  
 133-1B2 DY A I L  
 146-1H2 DY A L

Cons ----- V---K-LRGG V-F---P-- -TGK-----

551

Lcr .....AKM  
 Lla .....AKM  
 Lmi .....AKM  
 Pmi .....SKL  
 Ppy G...GKSKL  
 Lno .....SKL  
 Ppe1 GKSKSKAKL  
 Phg .....AKL  
 GR .....SKL  
 YG .....SKL  
 Ppe2 .....KSKL

49-7C6 TNG\*  
 78-0B10 TNG\*  
 90-1B5 TNG\*  
 133-1B2 TNG\*  
 146-1H2 TNG\*

FIG. 19A (Continued)

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Lcr:	Luciola cruciata	Phg:	Phengodes sp.
Lla:	Luciola lateralis	Lmi:	Luciola mingrelia
Gr:	Pyrophorus plagiophthalmus (green)		
YG:	Pyrophorus plagiophthalmus (yellow green)		
Pmi:	Pyrocoelia miyako	Ppy:	Photinus pyralis
Lno:	Lampyris noctiluca		
Ppe-2:	Photuris pennsylvanica (2)		
Ppe-1:	Photuris pennsylvanica (1)		
Cons:	Sites which are strictly conserved		

FIG. 19A (Continued)

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1						50
Lcr	MENMENDE.N	IVVGPKPFYP	IEEGSAGTQL	RKYMERYAKL	.GAIAPTNAV	
Lla	MENMENDE.N	IVYGPEPFYP	IEEGSAGAQL	RKYMDRYAKL	.GAIAPTNAV	
Lmi	ME.MEKKEE.N	VVYGPLPFYP	IEEGSAGIQL	HKYMHQYAKL	.GAIAPSNAL	
Pmi	...MEDDSKH	IMHGHRSIL	WEDGTAGEQL	HKAMKRYAQV	PGTIAFTDAH	
Ppy	...MED.AKN	IKKGPAFPYP	LEDGTAGEQL	HKAMKRYALV	PGTIAFTDAH	
Lno	...MED.AKN	IMHGPAFPYP	LEDGTAGEQL	HKAMKRYAQV	PGTIAFTDAH	
Ppe1	...MSI.ENN	ILIGPPPYYP	LEEGTAGEQL	HRAISRYAAV	PGTLAYTDVH	
Ppe2	...MED..KN	ILYGPEPFYP	LADGTAGEQM	FYALSRYADI	SGCIALTNAH	
Phg	MIKME..EEH	VMPGAMPRL	LFEGTAGQQL	HRALYKHSYF	PE..AIVDSH	
GR	...MMKREKN	VVYGPEPLHP	LEDLTAGEML	FRALRKHSHL	PQ..ALVDVY	
YG	...MMKREKN	VIYGPEPLHP	LEDLTAGEML	FRALRKHSHL	PQ..ALVDVF	
30-4B02	...MMKREKN	VIYGPEPLHP	LEDLTAGEML	FRALRKHSHL	PQ..ALVDVY	
81-6G01	...MMKREKN	VIYGPEPLHP	LEDLTAGEML	FRALRKHSHL	PQ..ALVDVY	
Cons	---M-----	---G-----	-----AG---	-----	-----A-----	
51						100
Lcr	TGVDYSYAEY	LEKSCCLGKA	LQNYGLVVDG	RIALCSENCE	EFFIPVIAAGL	
Lla	TGVDYTYAEY	LEKSCCLGRA	LKNYGLVVDG	RIALCSENCE	EFFIPVLAGL	
Lmi	TGVDISYQY	FDITCRLAE	MKNFGMKPEE	HIALCSENCE	EFFIPVLAGL	
Pmi	AEVNITYSEY	FEMSCRLAET	MKRYGLGLQH	HIAVCSETSL	QFFMPVCGAL	
Ppy	IEVNITYAEY	FEMSVRLAEA	MKRYGLNTNH	RIVVCSENSL	QFFMPVILGAL	
Lno	AEVNITYSEY	FEMACRLAET	MKRYGLGLQH	HIAVCSENSL	QFFMPVCGAL	
Ppe1	TELEVITYKEF	LDVTCRLAEA	MKNYGLGLQH	TISVCSENCV	QFFMPICAAAL	
Ppe2	TKENVLYEEF	LKLSCLRLAE	PKKYGLKQND	TIAVCSENGL	QFFLPPLIASL	
Phg	THEIISYAKI	LDMSCLRLAVS	FQKYGLTQNN	IIGICSENNL	NFFNPVIAAF	
GR	GEEWISYKEF	FETTCLLAQS	LHNCGYKMSD	VVSICAENNK	RFFVPPIAAW	
YG	GDESLSYKEF	FEATCLLAQS	LHNCGYKMND	VVSICAENNK	RFFIPIIAAW	
30-4B02	GDESLSYKEF	FEATVLLAQS	LHNCGYKMND	VVSICAENNK	RFFIPVIAAW	
81-6G01	GDESLSYKEF	FEATVLLAQS	LHNCGYKMND	VVSICAENNT	RFFIPVIAAW	
Cons	-----Y---	-----L---	-----G-----	-----C-E---	-----FF-P-----	
101						150
Lcr	FIGVGVAPT	EIYTLREL	SLGISKPTIV	FSSKKGLDKV	ITVQKTVTI	
Lla	FIGVGVAPT	EIYTLREL	SLGISKPTIV	FSSKKGLDKV	ITVQKTVATI	
Lmi	YIGVAVAPT	EIYTLREL	SLGIAQPTIV	FSSRKGLPKV	LEVQKTVTI	
Pmi	FIGVGVAPT	DIYNERELYN	SLFISQPTIV	FCSKRALQKI	LGVQKKLPVI	
Ppy	FIGVAVAPT	DIYNERELYN	SMNISQPTIV	FVSKKGLQKI	LNVQKKLPVI	
Lno	FIGVGVAPT	DIYNERELYN	SLSISQPTIV	SCSKRALQKI	LGVQKKLPVI	
Ppe1	YGVVATAPT	DIYNERELYN	SLSISQPTIV	FTSRNSLQKI	LGVQSRPLPI	
Ppe2	YLGIIAAPVS	DKYIERELIH	SLGIVKPRII	FCSKNTFQKV	LNVKSKLKVY	
Phg	YLGITVATV	DTYTDRELSE	TLNITKQML	FCSKQSLPIV	MKTMKIMPVY	
GR	YIGMIVAPV	EGYIPDELCK	VMGISRPQLV	FCTKNILNKV	LEVQSRDTFI	
YG	YIGMIVAPV	ESYIPDELCK	VMGISKPQIV	FCTKNILNKV	LEVQSRNTFI	
30-4B02	YIGMIVAPV	ESYIPDELCK	VMGISKPQIV	FTTKNILNKV	LEVQSRNTFI	
81-6G01	YIGMIVAPV	ESYIPDELCK	VMGISKPQIV	FTTKNILNKV	LEVQSRNTFI	
Cons	--G---A---	--Y---EL--	---I---P---	-----	-----	

FIG. 19B

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	151				200
Lcr	KTIVILDSKV	DYRGYQCLDT	FIKRNTPPGF	QASSFKTVEV	.DRKEQVALI
Lla	KTIVILDSKV	DYRGYQSM DN	FIKKNTPQGF	KGSSFKTVEV	.NRKEQVALI
Lmi	KKIVILDSKV	NFGGHDCMET	FIKKHVELGF	QPSSFVPIDV	.KNRKQHVALI
Pmi	QKIVILDSRE	DYMGKQSMYS	FIESHLPAGF	NEYDYIPDSF	.DRETATALI
Ppy	QKIIIMDSKT	DYQGFQSMYT	FVTSHLPPGF	NEYDFVPESF	.DRDKTIALI
Lno	QKIVILDSRE	DYMGKQSMYS	FIESHLPAGF	NEYDYIPDSF	.DRETATALI
Ppe1	KKIIILDGKK	DYLGYSMQS	FMKEHVPANF	NVSAPKPLSF	.DLDR.VACI
Ppe2	ETIIILD LNE	DLGGYQCLNN	FISQNSDINL	DVKKFKPNSF	.NRDDQVALV
Phg	QKLLIIDS MQ	DIGGIECVHS	FVSRYTDEHF	DPLKFVPLDF	.DPREQVALI
GR	KRIIILDAVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHY	.DPVEQVA AI
YG	KRIIILDTVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHY	.DPVEQVA AI
30-4B02	KRIIILDTVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHY	.DPVEQVA AI
81-6G01	KRIIILDTVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHF	.DPVEQVA AI
Cons	----I-D---	---G-----	F-----	-----	-----A--

	201				250
Lcr	MNSSSGSTGLP	KGVLTHENT	VTRFSHARDP	IYGNQVSPGT	AVLTVVPFHH
Lla	MNSSSGSTGLP	KGVLTHENA	VTRFSHARDP	IYGNQVSPGT	AILTVVPFHH
Lmi	MNSSSGSTGLP	KGVRITHEGA	VTRFSHAKDP	IYGNQVSPGT	AILTVVPFHH
Pmi	MNSSSGSTGLP	KGVDLTHMNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH
Ppy	MNSSSGSTGLP	KGVALPHRTA	CVRFSHARDP	IFGNQIIPDT	AILSVVPFHH
Lno	MNSSSGSTGLP	KGVELTHQNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH
Ppe1	MNSSSGSTGLP	KGVPISHRNT	IYRFSHCRDP	VFGNQIIPDT	TILCAVPFHH
Ppe2	MFSSGTTGVS	KGVM LTHKNI	VARFSHCKDP	TFGNAINPTT	AILTVIPFHH
Phg	MTSSGTTGLP	KGVM LTHRNI	CVRFVHSRDP	LFGTRFIPET	SILSLVPFHH
GR	LCSSGTTGLP	KGVMQTHRNV	CVRLIHALDP	RVGTQLIPGV	TVLVYLPFFH
YG	LCSSGTTGLP	KGVMQTHQNI	CVRLIHALDP	RAGTQLIPGV	TVLVYLPFFH
30-4B02	LCSSGTTGLP	KGVMQTHQNI	CVRLIHALDP	RAGTQLIPGV	TVLVYLPFFH
81-6G01	LCSSGTTGLP	KGVMQTHQNI	CVRLIHALDP	RAGTQLIPGV	TVLVYLPFFH
Cons	--SSG-TG--	KGV---H---	--R--H--DP	--G----P--	--L---PF-H

	251				300
Lcr	GFGMFTTLGY	LICGFRVVML	TKFDEETFLK	TLQDYKCTSV	ILVPTLFAIL
Lla	GFGMFTTLGY	LTCGFRIVML	TKFDEETFLK	TLQDYKCSSV	ILVPTLFAIL
Lmi	GFGMFTTLGY	FACGYRVVML	TKFDEELFLR	TLQDYKCTSV	ILVPTLFAIL
Pmi	VFQMFTTLGY	LTCGFRIVLM	YRFEBELFLR	SLQDYKIQSA	LLVPTLFSFF
Ppy	GFGMFTTLGY	LICGFRVVML	YRFEBELFLR	SLQDYKIQSA	LLVPTLFSFF
Lno	GFGMFTTLGY	LTCGFRIVLM	YRFEBELFLR	SLQDYKIQSA	LLVPTLFSFF
Ppe1	AFGTFTNLGY	LICGFHVVLN	YRFNEHLFLQ	TLQDYKCQSA	LLVPTVLAFL
Ppe2	GFGMTTTLGY	FTCGFRVALM	HTFBEKLFLQ	SLQDYKVEST	LLVPTLMAFF
Phg	AFGMFTTLSY	FIVGLKIVMM	KRFDGELFLK	TIQNYKIPTI	VIAPPVMVFL
GR	AFGFSINLGY	FMVGLRVIML	RRFDQEAFLK	AIQDYEVRSV	INVPAIILFL
YG	AFGFSINLGY	FMVGLRVIML	RRFDQEAFLK	AIQDYEVRSV	INVPAIILFL
30-4B02	AFGFSINLGY	FMVGLRVIML	RRFDQEAFLK	AIQDYEVRSV	INVPAIILFL
81-6G01	AFGFSITLGY	FMVGLRVIMF	RRFDQEAFLK	AIQDYEVRSV	INVPSVILFL
Cons	-F-----L-Y	---G-----	--F----FL-	--Q-Y-----	---P-----

FIG. 19B (Continued)

SUBSTITUTE SHEET (RULE 26)

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301		350	
Lcr	NKSELLNKYD	LSNLVEIASG	GAPLSKEVGE
Lla	NRSELLDKYD	LSNLVEIASG	GAPLSKEIGE
Lmi	NKSELIDKFD	LSNLTEIASG	GAPLAKEVGE
Pmi	AKSTLVDKYD	LSNLHEIASG	GAPLAKEVGE
Ppy	AKSTLIDKYD	LSNLHEIASG	GAPLSKEVGE
Lno	AKSTLVDKYD	LSNLHEIASG	GAPLAKEVGE
Ppe1	AKNPLVDKYD	LSNLHEIASG	GAPLSKEISE
Ppe2	AKSALVEKYD	LSHLKEIASG	GAPLSKEIGE
Phg	AKSHLVDKYD	LSSIKEIATG	GAPLGPALAN
GR	SKSPLVDKYD	LSSLRELCCG	AAPLAKEVAE
YG	SKSPLVDKYD	LSSLRELCCG	AAPLAKEVAE
30-4B02	SKSPLVDKYD	LSSLRELCCG	AAPLAKEVAE
81-6G01	SKSPLVDKYD	LSSLRELCCG	AAPLAKEVAE
Cons	----L--K-D	LS---E---G	-APL-----
351		400	
Lcr	TSALIIITPEG	DDKPGASGKV	VPLFKAKVID
Lla	TSALIIITPEG	DDKPGASGKV	VPLFKAKVID
Lmi	TSALIIITPEG	DDKPGASGKV	VPLFKVKVID
Pmi	TSALIIITPEG	DDKPGACGKV	VPFETAIVD
Ppy	TSALIIITPEG	DDKPGAVGKV	VPFFFAKVVD
Lno	TSALIIITPEG	DDKPGACGKV	VPFFSAKIVD
Ppe1	TCAIVITABG	EFKLGAVGKV	VPFYSKVLVD
Ppe2	TSAVLITPDT	DVRPGSTGKI	VPFHAVKVVD
Phg	CAVLITPHN	KIKTGSTGQV	LPYVTAKIVD
GR	TSANIHSLRD	EFKSGSLGRV	TPLMAAKIAD
YG	TSANIHSLRD	EFKSGSLGRV	TPLMAAKIAD
30-4B02	TSANIHSLRD	EFKSGSIGRV	TPLMAAKIAD
81-6G01	TSANIHSLRD	EFKSGSLGRV	TPLMAAKIAD
Cons	--A-----	----G--G--	-P----K--D
401		450	
Lcr	MLMKGYVNNP	EATKELIDEE	GWLHTGDIGY
Lla	MLMKGYVDNP	EATREIIDEE	GWLHTGDIGY
Lmi	SLMLGYSNNP	EATRETIDEE	GWLHTGDIGY
Pmi	MIMKGYVNNP	EATNALIDKD	GWLHSGDIAY
Ppy	MIMSGYVNNP	EATNALIDKD	GWLHSGDIAY
Lno	MIMKGYVNNP	EATSALIDKD	GWLHSGDIAY
Ppe1	MIMKGYINNP	EATRELIDEE	GWIHSGDIGY
Ppe2	MIMKSYNNNE	EATKAIINKD	GWLRSGLDIAY
Phg	IIMKGYQQNE	EETRLVIDKD	GWLHSGDIGY
GR	MVSKGYVNNV	EATKEAIDDD	GWLHSGDFGY
YG	MVSKGYVNNV	EATKEAIDDD	GWLHSGDFGY
30-4B02	MVSKGYVNNV	EATKEAIDDD	GWLHSGDFGY
81-6G01	MVSKGYVNNV	EATKEAIDDD	GWLHSGDFGY
Cons	-----Y--N-	E-T---I---	GW---GD--Y

FIG. 19B (Continued)

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	451				500
Lcr	GYQVPPAELE	SVLLQHPSIF	DAGVAGVPDP	VAGELPGAVV	VLESGKNMTE
Lla	GYQVPPAELE	SVLLQHNPNI	DAGVAGVPDP	IAGELPGAVV	VLEKGSMTTE
Lmi	GYQVPPAELE	SVLLQHNPNI	DAGVAGVPDP	DAGELPGAVV	VMEKGKTMTE
Pmi	GYQVPPAELE	SILLQHPPFI	DAGVAGIPDP	DAGELPAAVV	VLEEGKMMTE
Ppy	GYQVAPAELE	SILLQHNPNI	DAGVAGLPDD	DAGELPAAVV	VLEHGKTMTE
Lno	GYQVPPAELE	SILLQHPPFI	DAGVAGIPDP	DAGELPAAVV	VLEEGKTMTE
Ppe1	GYQVPPAELE	ALLLQHPPFI	DAGVAGVPDE	VAGDLPGAVV	VLKEGKSITE
Ppe2	GYQVAPAEIE	GILLQHPPYIV	DAGVTGIPDE	AAGELPAAV	VVQTGKYLNE
Phg	AYQVAPAELE	ALLLQHPPYIA	DAGVTGIPDE	EAGELPAAV	VLEPGKTMTE
GR	GSQVAPAELE	EILLKNPCIR	DVAVVGIPDL	EAGELPSAFV	VIQPGKEITA
YG	GSQVAPAELE	EILLKNPCIR	DVAVVGIPDL	EAGELPSAFV	VKQPGKEITA
30-4B02	GSQVAPAELE	EILLTNPCIR	DVAVVGIPDL	EAGELPSAFV	VKQPGKEITA
81-6G01	GSQVAPAELE	EILLKNPCIR	DVAVVGIPDL	EAGELPSAFV	VKQPGKEITA
Cons	--QV-PAE-E	--LL--P-I-	D--V-G-PD-	--AG-LP-A-V	V---GK----

	501				550
Lcr	KEVMDYVASQ	VSNAKRLRGG	VRFDVDEVPKG	LTGKIDGRA.	IREILKKPV.
Lla	KEVMDYVASQ	VSNAKRLRGG	VRFDVDEVPKG	LTGKIDGKA.	IREILKKPV.
Lmi	KEIVDYVNSQ	VVNHKRLRGG	VRFDVDEVPKG	LTGKIDAKV.	IREILKKPQ.
Pmi	QEVMDYVAGQ	VTASKRLRGG	VKFDVDEVPKG	LTGKIDSRK.	IREILTMGQK
Ppy	KEIVDYVASQ	VTAKKLRRGG	VVFDVDEVPKG	LTGKIDARK.	IREILIKAKK
Lno	QEVMDYVAGQ	VTASKRLRGG	VKFDVDEVPKG	LTGKIDGRK.	IREILMMGKK
Ppe1	KEIQDYVAGQ	VTSSKKLRGG	VEFVKEVPKG	FTGKIDTRK.	IKEILIKAQK
Ppe2	QIVQNFVSSQ	VSTAKWLRGG	VKFLDEIPKG	STGKIDRKV.	LRQMFEXH..
Phg	KEVMDYIAER	VTPTKRLRGG	VLFDVNNIPKG	ATGKLV RTE.	LRRLLTQRA.
GR	KEVYDYLAE	VSHTKYLRGG	VRFDVDSIPRN	VTGKITRTEL	LKQLLEKS..
YG	KEVYDYLAE	VSHTKYLRGG	VRFDVDSIPRN	VTGKITRTEL	LKQLLEKS..
30-4B02	KEVYDYLAE	VSHTKYLRGG	VRFDVDSIPRN	VTGKITRTEL	LKQLLEK...
81-6G01	KEVYDYLAE	VSHTKYLRGG	VRFDVDSIPRN	VTGKITRTEL	LKQLLEK...
Cons	-----	V---K-LRGG	V-F----P--	-TGK-----	-----

	551
Lcr	.....AKM
Lla	.....AKM
Lmi	.....AKM
Pmi	.....SKL
Ppy	G...GKSKL
Lno	.....SKL
Ppe1	GKSKSKAKL
Ppe2	.....KSKL
Phg	.....AKL
GR	.....SKL
YG	.....SKL
30-4B02	.....AGG*
81-6G01	.....AGG*
Cons	-----K-

FIG. 19B (Continued)



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1	50
Lcr	MENMENDE.N IVVGPKPFYP IEEGSAGTQL RXYMERYAKL .GAIIFTNAV
Lla	MENMENDE.N IVYGPEPFYP IEEGSAGAQL RXYMDRYAKL .GAIIFTNAL
Lmi	ME.MEKKE.N VVYGPLPFYP IEEGSAGIQL HKYMHQYAKL .GAIAFSNAL
Pmi	...MEDDSKH IMHGHRSIL WEDGTAGEQL HKAMKRYAQV PGTFIAFTDAH
Ppy	...MED.AKN IKKGPAFPYP LEDGTAGEQL HKAMKRYALV PGTFIAFTDAH
Lno	...MED.AKN IMHGPAFPYP LEDGTAGEQL HKAMKRYAQV PGTFIAFTDAH
Ppe1	...MSI.ENN ILIGPPPYYP LEEGTAGEQL HRAISRYAAV PGTLAYTDVH
Ppe2	...MED..KN ILYGPEPFYP LADGTAGEQM FYALSRYADI SGCIALTNAH
Phg	MIKME..EEH VMPGAMPRLD LFEGTAGOQL HRALYKHSYF PE..AIVDSH
YG	...MMKREKN VIYGPEPLHP LEDLTAGEML FRALRKHSHL PQ..ALVDVF
---XX00000 X00-000X0 OOX0--OX0 X00X0XX00 OX00-XX0XX	
51	100
Lcr	TGVDYSYAEY LEKSCCLGKA LQNYGLVVDG RIALCSENCE EFFIPVLAGL
Lla	TGVDYTYAEY LEKSCCLGEA LKNYGLVVDG RIALCSENCE EFFIPVLAGL
Lmi	TGVDISYQY FDITCRLAEA MKNFGMKPEE HIALCSENCE EFFIPVLAGL
Pmi	AEVNITYSEY FEMSCRLAET MKRYGLGLQH HIAVCSETSL QFFMPVCGAL
Ppy	IEVNITYAEY FEMSVRLAEA MKRYGLNTNH RIVVCSENSL QFFMPVLGAL
Lno	AEVNITYSEY FEMACRLAET MKRYGLGLQH HIAVCSENSL QFFMPVCGAL
Ppe1	TELEVITYKEF LDVTCRLAEA MKNYGLGLQH TISVCSENCV QFFMPICAAAL
Ppe2	TKENVLYEEF LKLSCLRAES FKKYGLKQND TIAVCSENGL QFFLPLIASL
Phg	THEIISYAKI LDMSCLRAVS FQKYGLTQNN IIGICSENNL NFFNPVIAAF
YG	GDESLSYKEF FEATCLLAQS LHNCGYKMND VVSICAENNK RFFIPIIAAW
X0X0X--X X00X0X0X- XX0X-X0000 OX0X-X-00X X--O-X000X	
101	150
Lcr	FIGVGVAPT N EIYTLREL V H SLGISKPTIV FSSKKGLDKV ITVQKT VTTI
Lla	FIGVGVAPT N EIYTLREL V H SLGISKPTIV FSSKKGLDKV ITVQKT VATI
Lmi	YIGVAVAPT N EIYTLREL N H SLGIAQPTIV FSSRKGLPKV LEVQKT VTCI
Pmi	FIGVGVAPT N DIYNEREL Y N SLFISQPTIV FCSKRALQKI LGVQKKLPVI
Ppy	FIGVAVAPT N DIYNEREL L N SMNISQPTV FVSKKGLQKI LNVQKKLP II
Lno	FIGVGVAPT N DIYNEREL Y N SLSISQPTIV SCSKRALQKI LGVQKKLP II
Ppe1	YVG VATAPT N DIYNEREL Y N SLSISQPTIV FTSRNSLQKI LGVQSRLP II
Ppe2	YLGIIAAPVS DKYIEREL IH SLGIVKPRII FCSKNTFQKV LNVKSKLK YV
Phg	YLGITVATVN DTYTDRELSE TLNITKQML FCSKQSLPIV MKTMKIMP YV
YG	YIGMIVAPVN ESYIPDELCK VMGISKPTIV FCTKNILNKV LEVQSR TNFI
X0-X00-OX0 XX-OXX--00 X0-OX-X00 OOX0000X00 000-X0X00	

FIG. 19C

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151						200					
Lcr	KTIVILDSKV	DYRGYQCLDT	FIKRNTPPGF	QASSFKTVEV	.DRKEQVALI	Lcr	KTIVILDSKV	DYRGYQCLDT	FIKRNTPPGF	QASSFKTVEV	.DRKEQVALI
Lla	KTIVILDSKV	DYRGYQSMDN	FIKRNTPQGF	KGSSFKTVEV	.NRKEQVALI	Lla	KTIVILDSKV	DYRGYQSMDN	FIKRNTPQGF	KGSSFKTVEV	.NRKEQVALI
Lmi	KKIVILDSKV	NFGGHDCMET	FIKKHVELGF	QPSFVPIDV	KNRKQHVALI	Lmi	KKIVILDSKV	NFGGHDCMET	FIKKHVELGF	QPSFVPIDV	KNRKQHVALI
Pmi	QKIVILDSRE	DYMGKQSMYS	FIESHLPAGF	NEYDYIPDSF	.DRETATALI	Pmi	QKIVILDSRE	DYMGKQSMYS	FIESHLPAGF	NEYDYIPDSF	.DRETATALI
Ppy	QKIIIMDSKT	DYQGFQSMYT	FVTSHLPPGF	NEYDFVPESF	.DRDKTIALI	Ppy	QKIIIMDSKT	DYQGFQSMYT	FVTSHLPPGF	NEYDFVPESF	.DRDKTIALI
Lno	QKIVILDSRE	DYMGKQSMYS	FIESHLPAGF	NEYDYIPDSF	.DRETATALI	Lno	QKIVILDSRE	DYMGKQSMYS	FIESHLPAGF	NEYDYIPDSF	.DRETATALI
Ppe1	KKIIILDGKK	DYLGYSQMS	FMKEHVPANF	NVSAFKPLSF	.DLDR.VACI	Ppe1	KKIIILDGKK	DYLGYSQMS	FMKEHVPANF	NVSAFKPLSF	.DLDR.VACI
Ppe2	ETIIILDLE	DLGGYQCLNN	FISQNSDINL	DVKKFKPNSF	.NRDDQVALV	Ppe2	ETIIILDLE	DLGGYQCLNN	FISQNSDINL	DVKKFKPNSF	.NRDDQVALV
Phg	QKLLIIDSMQ	DIGGIECVHS	FVSRYTDEHF	DPLKFVPLDF	.DPREQVALI	Phg	QKLLIIDSMQ	DIGGIECVHS	FVSRYTDEHF	DPLKFVPLDF	.DPREQVALI
YG	KRIIILDTVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHY	.DPVEQVAAT	YG	KRIIILDTVE	NIHGCESLPN	FISRYSDGNI	A..NFKPLHY	.DPVEQVAAT
OXOX-O-XXO XXO-OXOXOO -OOOXOXOXX O--OOOOOXX -OXOOOO-XO											
201						250					
Lcr	MNSSGSTGLP	KGVLTHENT	VTRFSHARDP	IYGNQVSPGT	AVLTVVPFHH	Lcr	MNSSGSTGLP	KGVLTHENT	VTRFSHARDP	IYGNQVSPGT	AVLTVVPFHH
Lla	MNSSGSTGLP	KGVLTHENA	VTRFSHARDP	IYGNQVSPGT	AILTVVPFHH	Lla	MNSSGSTGLP	KGVLTHENA	VTRFSHARDP	IYGNQVSPGT	AILTVVPFHH
Lmi	MNSSGSTGLP	KGVRITHEGA	VTRFSHAKDP	IYGNQVSPGT	AILTVVPFHH	Lmi	MNSSGSTGLP	KGVRITHEGA	VTRFSHAKDP	IYGNQVSPGT	AILTVVPFHH
Pmi	MNSSGSTGLP	KGVDLTHMNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH	Pmi	MNSSGSTGLP	KGVDLTHMNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH
Ppy	MNSSGSTGLP	KGVALPHTA	CVRFSHARDP	IFGNQIIPDT	AILSVVPFHH	Ppy	MNSSGSTGLP	KGVALPHTA	CVRFSHARDP	IFGNQIIPDT	AILSVVPFHH
Lno	MNSSGSTGLP	KGVELTHQNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH	Lno	MNSSGSTGLP	KGVELTHQNV	CVRFSHCRDP	VFGNQIIPDT	AILTVIPFHH
Ppe1	MNSSGSTGLP	KGVPISHRNT	IYRFSHCRDP	VFGNQIIPDT	TILCAVPFHH	Ppe1	MNSSGSTGLP	KGVPISHRNT	IYRFSHCRDP	VFGNQIIPDT	TILCAVPFHH
Ppe2	MFSSGTTGVS	KGVMITHQNI	VARFSHCKDP	TFGNAINPTT	AILTVIPFHH	Ppe2	MFSSGTTGVS	KGVMITHQNI	VARFSHCKDP	TFGNAINPTT	AILTVIPFHH
Phg	MTSSGTTGLP	KGVMITHQNI	CVRFVHSRDP	LFGTRFIPET	SILSLVPFHH	Phg	MTSSGTTGLP	KGVMITHQNI	CVRFVHSRDP	LFGTRFIPET	SILSLVPFHH
YG	LCSSGTTGLP	KGVMQTHQNI	CVRLIHALDP	RAGTQLIPGV	TVLVLYLPFHH	YG	LCSSGTTGLP	KGVMQTHQNI	CVRLIHALDP	RAGTQLIPGV	TVLVLYLPFHH
XX---X--OO ---OXO-OOO OO-XX-OX-- OX-XOXO-OX XX-XXX--X-											
251						300					
Lcr	GFGMFTTLGY	LICGFRVVML	TKFDEETFLK	TLQDYKCTSV	ILVPTLFAIL	Lcr	GFGMFTTLGY	LICGFRVVML	TKFDEETFLK	TLQDYKCTSV	ILVPTLFAIL
Lla	GFGMFTTLGY	LTCGFRIVML	TKFDEETFLK	TLQDYKCSSV	ILVPTLFAIL	Lla	GFGMFTTLGY	LTCGFRIVML	TKFDEETFLK	TLQDYKCSSV	ILVPTLFAIL
Lmi	GFGMFTTLGY	FACGYRVVML	TKFDEELFLR	TLQDYKCTSV	ILVPTLFAIL	Lmi	GFGMFTTLGY	FACGYRVVML	TKFDEELFLR	TLQDYKCTSV	ILVPTLFAIL
Pmi	VFQMFTTLGY	LTCGFRIVLM	YRFEELFLR	SLQDYKIQSA	LLVPTLFSFF	Pmi	VFQMFTTLGY	LTCGFRIVLM	YRFEELFLR	SLQDYKIQSA	LLVPTLFSFF
Ppy	GFGMFTTLGY	LICGFRVVLV	YRFEELFLR	SLQDYKIQSA	LLVPTLFSFF	Ppy	GFGMFTTLGY	LICGFRVVLV	YRFEELFLR	SLQDYKIQSA	LLVPTLFSFF
Lno	GFGMFTTLGY	LTCGFRIVLM	YRFEELFLR	SLQDYKIQSA	LLVPTLFSFF	Lno	GFGMFTTLGY	LTCGFRIVLM	YRFEELFLR	SLQDYKIQSA	LLVPTLFSFF
Ppe1	AFGTFTNLGY	LICGFHVLM	YRFNEHLFLQ	TLQDYKCQSA	LLVPTVLAFL	Ppe1	AFGTFTNLGY	LICGFHVLM	YRFNEHLFLQ	TLQDYKCQSA	LLVPTVLAFL
Ppe2	GFGMITTLGY	FTCGFRVALM	HTFEELFLQ	SLQDYKVEST	LLVPTLMAFF	Ppe2	GFGMITTLGY	FTCGFRVALM	HTFEELFLQ	SLQDYKVEST	LLVPTLMAFF
Phg	AFGMFTTLSY	FIVGLKIVVM	KRFDGELFLK	TIQNYKIPTI	VIAPPVMVFL	Phg	AFGMFTTLSY	FIVGLKIVVM	KRFDGELFLK	TIQNYKIPTI	VIAPPVMVFL
YG	AFGFSINLGY	FMVGLRVIML	RRFDQEAFLK	AIQDYEVRSV	INVPAILFL	YG	AFGFSINLGY	FMVGLRVIML	RRFDQEAFLK	AIQDYEVRSV	INVPAILFL
X-OXXXX-O- XOX-XOXXXX OO-OXOX--O XX-O-XOOOO XXO-XXXXOO											

FIG. 19C (Continued)

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	301				350
Lcr	NKSELLNKYD	LSNLVEIASG	GAPLSKEVGE	AVARRFNLPG	VRQGYGLTET
Lla	NRSELLDKYD	LSNLVEIASG	GAPLSKEIGE	AVARRFNLPG	VRQGYGLTET
Lmi	NKSELIDKFD	LSNLTEIASG	GAPLAKEVGE	AVARRFNLPG	VRQGYGLTET
Pmi	AKSTLVDKYD	LSNLHEIASG	GAPLAKEVGE	AVAKRFKLPG	IRQGYGLTET
Ppy	AKSTLIDKYD	LSNLHEIASG	GAPLSKEVGE	AVAKRFHLPG	IRQGYGLTET
Lno	AKSTLVDKYD	LSNLHEIASG	GAPLAKEVGE	AVAKRFKLPG	IRQGYGLTET
Ppe1	AKNPLVDKYD	LSNLHEIASG	GAPLSKEISE	IAAKRFKLPG	IRQGYGLTET
Ppe2	AKSALVEKYD	LSHLKEIASG	GAPLSKEIGE	MVKKRFKLNF	VRQGYGLTET
Phg	AKSHLVDKYD	LSSIKEIATG	GAPLGALAN	AVAKRLKLG	IIQGYGLTET
YG	SKSPLVDKYD	LSSLRELCCG	AAPLAKEVAE	VAVKRLNLPG	IRCGFGLTES
	X000-00-0-	--X00-XXX-	X---X000X0	XXX0-XX-00	OOX-X----X
	351				400
Lcr	TSALIIITPEG	DDKPGASGKV	VPLFKAKVID	LDTKKS LGPN	RRGEVCVKGP
Lla	TSALIIITPEG	DDKPGASGKV	VPLFKAKVID	LDTKKT LGPN	RRGEVCVKGP
Lmi	TSALIIITPEG	DDKPGASGKV	VPLFKVKVID	LDTKKT LGVN	RRGEICVKGP
Pmi	TSALIIITPEG	DDKPGACGKV	VPFFTA KIVD	LDTGKT LGVN	QRGELCVKGP
Ppy	TSALIIITPEG	DDKPGAVGKV	VPFFEA KIVD	LDTGKT LGVN	QRGELCVKGP
Lno	TSALIIITPEG	DDKPGACGKV	VPFFSA KIVD	LDTGKT LGVN	QRGELCVKGP
Ppe1	TCAIVITAE	EFKLGA VGKV	VPFYSK VLD	LNTGKK LGPN	ERGEICFKGP
Ppe2	TSAVLITPDT	DVRPGSTGKI	VPFHAV KIVD	PTTGKILGPN	ETGELYFKGD
Phg	CCAVLITPHN	KIKTGSTGQV	LPYVTAKIVD	TKTGKNLGPN	QTGELCFKSD
YG	TSANIHSLGD	EFKSGSLGRV	TPLMAAKIAD	RETGKALGPN	QVGELCVKGP
	00-X0XXXXX	XXOX-X0-X0	X-XX00-XX-	XX-O-X--O-	OX--000000
	401				450
Lcr	MLMKGYVNNP	EATKELIDEE	GWLHTGDIGY	YDEEKHFFIV	DRLKSLIKYK
Lla	MLMKGYVDNP	EATREIIDEE	GWLHTGDIGY	YDEEKHFFIV	DRLKSLIKYK
Lmi	SLMLGYSNNP	EATRETIDEE	GWLHTGDIGY	YDEDEHFFIV	DRLKSLIKYK
Pmi	MIMKGYVNNP	EATNALIDKD	GWLHSGDIAY	YDKDGHFFIV	DRLKSLIKYK
Ppy	MIMSGYVNNP	EATNALIDKD	GWLHSGDIAY	WDEDEHFFIV	DRLKSLIKYK
Lno	MIMKGYVNNP	EATSALIDKD	GWLHSGDIAY	YDKDGHFFIV	DRLKSLIKYK
Ppe1	MIMKGYINNP	EATRELIDEE	GWIHSGDIGY	FDEDGHVYIV	DRLKSLIKYK
Ppe2	MIMKSYNNNE	EATKAIINKD	GWLRSGDIAY	YDNDGHFYIV	DRLKSLIKYK
Phg	IIMKGYYYQNE	EETRLVIDKD	GWLHSGDIGY	YDTDGNFHIV	DRLKELIKYK
YG	MVSKGYVNNV	EATKEAIDDD	GWLHSGDFGY	YDEDEHFFIV	DRYKELIKYK
	0XX00-00-X	-O-00X-0X0	--000--X0-	O-00X00XX-	--X-X-----

FIG. 19C (Continued)

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451		500	
Lcr	GYQVPPAELE SVLLQHPNIF	DAGVAGVPDP	VAGELPGAVV VLESGKNMTE
Lla	GYQVPPAELE SVLLQHPNIF	DAGVAGVPDP	IAGELPGAVV VLEKQKSMTE
Lmi	GYQVPPAELE SVLLQHPNIF	DAGVAGVPDP	DAGELPGAVV VMEKQKTMTE
Pmi	GYQVPPAELE SILLQHPFIF	DAGVAGIPDP	DAGELPAAVV VLEEGKMMTE
Ppy	GYQVAPAELE SILLQHPNIF	DAGVAGLPDD	DAGELPAAVV VLEHGKTMTE
Lno	GYQVPPAELE SILLQHPFIF	DAGVAGIPDP	DAGELPAAVV VLEEGKTMTE
Ppe1	GYQVPPAELE ALLLQHPFIE	DAGVAGVPDE	VAGDLPGAVV VLKEGKSITE
Ppe2	GYQVAPAEIE GILLQHPYIV	DAGVTGIPDE	AAGELPAAGV VVQTGKYLNE
Phg	AYQVAPAELE ALLLQHPYIA	DAGVTGIPDE	EAGELPAACV VLEPGKTMTE
YG	GSQVAPAELE EILLKNPCIR	DVAVVGIPDL	EAGELPSAFV VKQPGKEITA
OX--X---O-		XO--XX-O-X	-XX-X-O--X O--O--X-X- -XXO--OXOX
501		550	
Lcr	KEVMDYVASQ VSNAKRLRGG	VRFVDEVPKG	LTGKIDGRA. IREILKKPV.
Lla	KEVMDYVASQ VSNAKRLRGG	VRFVDEVPKG	LTGKIDGKA. IREILKKPV.
Lmi	KHIVDYVNSQ VVNHKRLRGG	VRFVDEVPKG	LTGKIDAKV. IREILKKPQ.
Pmi	QEVMDYVAGQ VTASKRLRGG	VKFVDEVPKG	LTGKIDSRK. IREILTMGQK
Ppy	KHIVDYVASQ VTTAKRLRGG	VVFVDEVPKG	LTGKLDARK. IREILIKAKK
Lno	QEVMDYVAGQ VTASKRLRGG	VKFVDEVPKG	LTGKIDGRK. IREILMMGKK
Ppe1	KHIQDYVAGQ VTSSKILRGG	VEFVKEVPKG	FTGKIDTRK. IKEILIKAQK
Ppe2	QIVQNFVSSQ VSTAKWLRGG	VKFLDEIPKG	STGKIDRKV. LRQMFEEKH..
Phg	KEVMDYLAER VTPTKRLRGG	VLFVNNIPKG	ATGKLV RTE. LRRLLTQRA.
YG	KEVDYLAER VSHTKYL RGG	VRFVDSIPRN	VTGKITR KEL LKQLLEKS..
OOOXOOXOX		-OOO-X----	-O-OXX-XX X---OXOXOO XXXXOOOOOO
551			
Lcr	.....AKM		
Lla	.....AKM		
Lmi	.....AKM		
Pmi	.....SKL		
Ppy	G...GSKL		
Lno	.....SKL		
Ppe1	GKSKSKAKL		
Ppe2	.....KSKL		
Phg	.....AKL		
YG	.....SKL		
OOOOOXX-O			

FIG. 19C (Continued)

Key to beetle luciferase sequences:

Lcr	<i>Luciola cruciata</i>
Lla	<i>Luciola lateralis</i>
Lmi	<i>Luciola mingrelica</i>
Pmi	<i>Pyrocoelia miyako</i>
Ppy	<i>Photinus pyralis</i>
Lno	<i>Lampyris noctiluca</i>
Ppe1	<i>Photuris pennsylvanica</i> (1)
Ppe2	<i>Photuris pennsylvanica</i> (2)
Phg	<i>Phengodes</i> sp.
YG	<i>Pyrophorus plagiophthalmus</i> – yellow-green luminescence

FIG. 19D

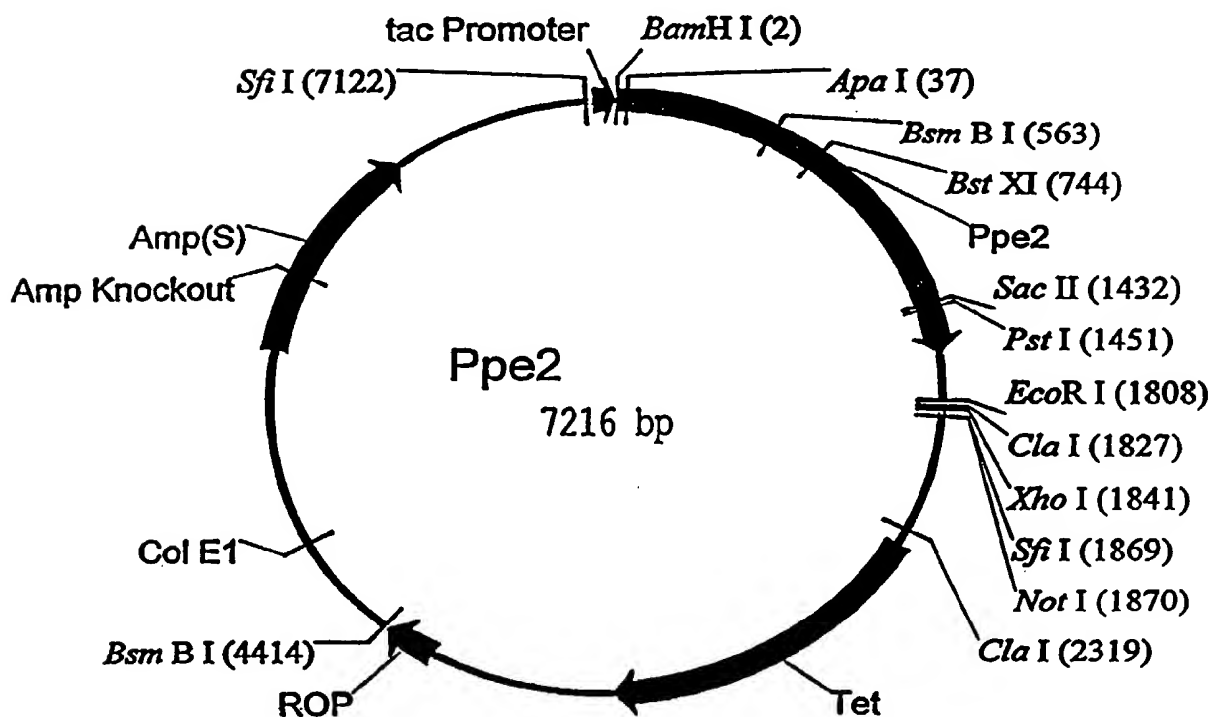


FIG. 20

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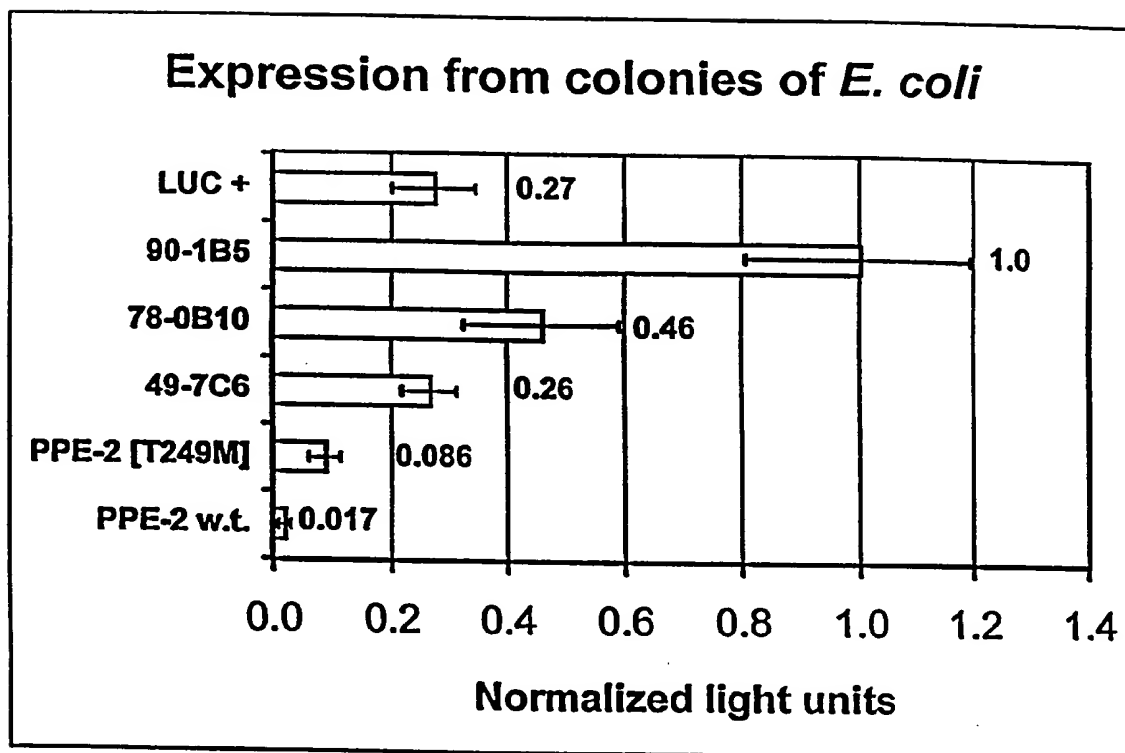


FIG. 21

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1uc49-7C6 (SEQ ID NO:1)

GGATCCAATG	GCAGATAAAA	ATATTTTATA	TGGGCCCGAA	CCATTTTATC	CCTTGGCTGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTTACGC	ATTATCTCGT	TATGCAGATA	TTTCAGGATG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGA	AAATGTTTTA	TATGAAGAGT	TTTTAAAATT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATGGTT	TGCAATTTTT	CCTTCCTATA	ATTGCATCAT	TGTATCTTGG	300
AATAATTGCA	GCACCTGTTA	GTGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAA	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TTAAAATATG	TAGAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTATCAAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTCC	GATATTAATC	TGGACGTAAA	540
AAAATTTAAA	CCAATATTCTT	TTAATCGAGA	CGATCAGGTT	GCGTTGGTAA	TGTTTTCTTC	600
TGGTACAAC	GGTGTTCGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTCTTCCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCAACGACAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GTTCTAATGC	ACACGTTTGA	AGAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTCTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACCT	AAAAGAAAAT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAACAATG	ACGTCAGACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTTCA	CGCTGTTAAA	GTGTGCGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AACCTGGAGA	ATTGTATTTT	AAAGGCGACA	TGATAATGAA	1200
AGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	AACAAAGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCATTAATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACRACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	CCGGATGAAG	CCGCGGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AAATTTTGTT	TCCAGTCAAG	TTTCAACAGC	CAAAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	ACCAATGGG					1639

FIG. 22

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luc49-6C10 (SEQ ID NO:2)

GGATCCAATG	GAAGATAAAA	ATATTTTATA	TGGACCTGAA	CCATTTTATC	CCTTGGCTGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTTACGC	ATTATCTCGT	TATGCAGATA	TTTCAGGATG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGC	CCCTGTTTTA	TATGAAGAGT	TGTTAAAATT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAAATGGTT	TGCAATTTTT	CCTTCCTATA	ATTGCATCAT	TGTATCTTGG	300
AATAATTGCA	GCACCTGTTA	GTGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAA	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TTAAAATATG	TAGAAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTCC	GATATTAATC	TGGACGTAAA	540
AAAATTTAAA	CCATATTCTT	TTAATCGAGA	CGATCAGGTT	GCGTTGGTAA	TGTTTTCTTC	600
TGGTACAAC	GGTGTTCGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTCATGCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCAACGACAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GTTCTAATGC	ACACGTTTGA	AGAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTTTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAATT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAACAATG	ACGTCAGACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTTCA	CGCTGTTAAA	GTTGTGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AAACTGGAGA	ATTGTATTTT	AAAGGCGACA	TGATAATGAA	1200
AGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	AACAAAGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCATTAATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	COGGATGAAG	CCGCGGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AAATTTTGTT	TCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	ACCAATGGG					1639

FIG. 23



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luc49-0G12 (SEQ ID NO:3)

GGATCCAATG	GAAGATAAAA	ATATTTTATA	TGGACCTGAA	CCATTTTATC	CCTTGGCTGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTACGC	ATTATCTCGT	TATGCAGATA	TTTCAGGATG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGC	CCCTGTTTTA	TATGAAGAGT	TTTTAAAATT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATGGTT	TGCAATTTTT	CCTTCCTATA	ATTGCATCAT	TGTATCTTGG	300
AATAATTGCA	GCACCTGTTA	GTGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAA	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TTAAAATATG	TAGAAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTCC	GATATTAATC	TTGACGTAAA	540
AAAATTTAAA	CCATATTCTT	TTAATCGAGA	CGATCAGGTT	GCGTTGGTAA	TGTTTTCTTC	600
TGGTACAAC	GGTGTTCGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGTACGATT	660
TTCTTATGCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCAACGACAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCGGAGTT	GTTCTAATGC	ACACGTTTGA	AGAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTCTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAAAT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAACAATG	ACGTCAGACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTTCA	CGCTGTTAAA	GTGTGCGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AAACTGGAGA	ATTGTATTTT	AAAGGCGACA	TGATAATGAA	1200
AGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	AACAAAGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCATTAATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	CCGGATGAAG	CCGCGGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AAATTTTGTT	TCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	<u>ACCAATGGG</u>					1639

FIG. 24

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1uc49-7A5 (SEQ ID NO:4)

GGATCCAATG	GAAGATAAAA	ATATTTTATA	TGGACCTGAA	CCATTTTATC	CCTTGGCTGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTTACGC	ATTATCTCGT	TATGCAGATA	TTTCAGGATG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGC	CCCTGTTTTA	TATGAAGAGT	TTTTAAAATT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATGGTT	TGCAATTTTT	CCTTCCTATA	ATTGCATCAT	TGTATCTTGG	300
AATAATTGCA	GCACCTGTTA	GTGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAA	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TAAGTGAATGT	420
AAAATCTAAA	TTAAAATATG	TAGAAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTCC	GATATTAATC	TTGACGTAAA	540
AAAATTTAAA	CCAATTTCTT	TTAATCGAGA	CGATCAGGTT	GCGTTGGTAA	TGTTTTCTTC	600
TGGTACAAC	GGTGTTCGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTATTGCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCAACGACAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GTTCTAATGC	ACACGTTTGA	AGAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTTTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAATT	GCATCTGGTG	GCGCACCTTT	960
ATCRAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAACAATG	ACGTCAGACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTTC	CGCTGTTAAA	GTTGTCGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AAACTGGAGA	ATTGTATTTT	AAAGGCGACA	TGATAATGAA	1200
AGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	AACAAAGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCATTAATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	CCGGATGAAG	CCGCGGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AAATTTTGTT	TCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	ACCAATGGG					1639

FIG. 25

luc49-4G11 (SEQ ID NO:5)

GGATCCAATG	GAAGATAAAA	ATATTTTATA	TGGACCTGAA	CCATTTTATC	CCTTGGCTGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTGACGC	ATTATCTCGT	TATGCAGATA	TTTCAGGATG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGC	CCCTGTTTTA	TATGAAGAGT	TGTTAAAATT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATGGTT	TGCAATTTTT	CCTTCCTATA	ATTGCATCAT	TGTATCTTGG	300
AATAATTGCA	GCACCTGTTA	GTGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAA	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TTAAAATATG	TAGAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTCC	GATATTAATC	TTGACGTAAA	540
AAAATTTAAA	CCATATTCTT	TTAATCGAGA	CGATCAGGTT	GCGTTGGTAA	TGTTTTCTTC	600
TGGTACAAC	GGTGTTCGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTCATGCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCAACGACAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GTTCTAATGC	ACACGTTTGA	AGAAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTTTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAAAT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTTAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAACAATG	ACGTCAGACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTTCA	CGCTGTTAAA	GTTGTGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AACTGGGAGA	ATTGTATTTT	AAAGGCGACA	TGATAATGAA	1200
AGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	AACAAAGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAAATG	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCATTAATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	CCGGATGAAG	CCGCGGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AAATTTTGTT	TCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	ACCAATGGG					1639

FIG. 26

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luc49-7C6 (SEQ ID NO:14)

		M	A	D	K	N	I	L	Y	G	P	E	P	F	Y	P	L	A	D	20
G	T	A	G	E	Q	M	F	Y	A	L	S	R	Y	A	D	I	S	G	C	40
I	A	L	T	N	A	H	T	K	E	N	V	L	Y	E	E	F	L	K	L	60
S	C	R	L	A	E	S	F	K	K	Y	G	L	K	Q	N	D	T	I	A	80
V	C	S	E	N	G	L	Q	F	F	L	P	I	I	A	S	L	Y	L	G	100
I	V	K	P	R	I	I	F	C	S	K	N	T	F	Q	K	V	L	N	V	120
K	S	K	L	K	Y	V	E	T	I	I	N	L	D	L	N	E	D	L	V	140
G	Y	Q	C	L	N	N	F	I	S	Q	N	S	D	I	N	L	D	V	K	160
K	F	K	P	Y	S	F	N	R	D	D	Q	V	A	L	V	M	F	S	S	180
G	T	T	G	V	S	K	G	V	M	L	T	H	K	N	I	V	A	R	F	200
S	L	A	K	D	P	T	F	G	N	A	I	N	P	T	T	A	I	L	T	220
V	I	P	F	H	H	G	F	G	M	M	T	T	L	G	Y	F	T	C	G	240
F	R	V	Y	L	M	H	T	F	E	E	K	L	F	L	Q	S	L	Q	D	260
Y	K	V	E	S	T	L	L	V	P	T	L	M	A	F	L	A	K	S	A	280
L	V	E	K	Y	D	L	S	H	L	K	E	I	A	S	G	G	A	P	L	300
S	K	E	I	G	E	M	V	K	K	R	F	K	L	N	F	V	R	Q	G	320
Y	G	L	T	E	T	T	S	A	V	L	I	T	P	N	N	D	V	R	P	340
G	S	T	G	K	I	V	P	F	H	A	V	K	V	V	D	P	T	T	G	360
K	I	L	G	P	N	E	P	G	E	L	Y	F	K	G	D	M	I	M	K	380
G	Y	Y	N	N	E	E	A	T	K	A	I	I	N	K	D	G	W	L	R	400
S	G	D	I	A	Y	Y	D	N	D	G	H	F	Y	I	V	D	R	L	K	420
S	L	I	K	Y	K	Y	G	Y	Q	V	A	P	A	E	I	E	G	I	L	440
Q	H	P	Y	I	V	D	A	G	V	T	G	K	Y	L	N	E	A	G	E	460
L	P	A	A	G	V	V	V	Q	T	G	K	Y	L	N	E	Q	I	V	Q	480
N	F	V	S	S	Q	V	S	T	A	K	W	L	R	G	G	V	K	F	L	500
D	E	I	P	K	G	S	T	G	K	I	D	R	K	V	L	R	Q	M	F	520
E	K	H	T	N	G															546

FIG. 27

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Luc49-6C10 (SEQ ID NO:15)

M E D K N I L Y G P E P F Y P L A D 20  
G T A G E Q M F Y A L S R Y A D I S G C 40  
I A L T N A H T K E N V L Y E E L L K L 60  
S C R L A E S F K K Y G L K Q N D T I A 80  
V C S E N G L Q F F L P I I A S L Y L G 100  
I I A A P V S D K Y I E R E L I H S L G 120  
I V K P R I I F C S K N T F Q K V L N V 140  
K S K L K Y V E T I I I L D L N E D L G 160  
G Y Q C L N N F I S Q N S D I N L D V K 180  
K F K P Y S F N R D D Q V A L V M F S S 200  
G T T G V S K G V M L T H K N I V A R F 220  
S H A K D P T F G N A I N P T T A I L T 240  
V I P F H H G F G M M T T L G Y F T C G 260  
F R V Y L M H T F E E K L F L Q S L Q D 280  
Y K V E S T L L V P T L M A F F A K S A 300  
L V E K Y D L S H L K E I A S G G A P L 320  
S K E I G E M V K K R F K L N F V R Q G 340  
Y G L T E T T S A V L I T P N N D V R P 360  
G S T G K I V P F H A V K V V D P T T G 380  
K I L G P N E T G E L Y F K G D M I M K 400  
G Y Y N N E E A T K A I I N K D G W L R 420  
S G D I A Y Y D N D G H F Y I V D R L K 440  
S L I K Y K G Y Q V A P A E I E G I L L 460  
Q H P Y I V D A G V T G I P D E A A G E 480  
L P A A G V V V Q T G K Y L N E Q I V Q 500  
N F V S S Q V S T A K W L R G G V K F L 520  
D E I P K G S T G K I D R K V L R Q M F 540  
E K H T N G 546

FIG. 28

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Luc49-OG12 (SEQ ID NO:16)

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      M E D K N I L Y G P E P F Y P L A D 20
G T A G E Q M F Y A L S R Y A D I S G C 40
I A L T N A H T K E N V L Y E E F L K L 60
S C R L A E S F K K Y G L K Q N D T I A 80
V C S E N G L Q F F L P I I A S L Y L G 100
I I A A P V S D K Y I E R E L I H S L G 120
I V K P R I I F C S K N T F Q K V L N V 140
K S K L K Y V E T I I I L D L N E D L G 160
G Y Q C L N N F I S Q N S D I N L D V K 180
K F K P Y S F N R D D Q V A L V M F S S 200
G T T G V S K G V M L T H K N I V V R F 220
S L A K D P T F G N A I N P T T A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V V L M H T F E E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F F A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P N N D V R P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E T G E L Y F K G D M I M K 400
G Y Y N N E E A T K A I I T K D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
N F V S S Q V S T A K W L R G G V K F L 520
D E I P K G S T G K I D R K V L R Q M F 540
E K H T N G 546

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FIG. 29

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Luc49-7A5 (SEQ ID NO:17)

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      M E D K N I L Y G P E P F Y P L A D   20
G T A G E Q M F Y A L S R Y A D I S G C   40
I A L T N A H T K E N V L Y E E F L K L   60
S C R L A E S F K K Y G L K Q N D T I A   80
V C S E N G L Q F F L P I I A S L Y L G  100
I I A A P V S D K Y I E R E L I H S L G  120
I V K P R I I F C S K N T F Q K V L N V  140
K S K L K Y V E T I I I L D L N E D L G  160
G Y Q C L N N F I S Q N S D I N L D V K  180
K F K P Y S F N R D D Q V A L V M F S S  200
G T T G V S K G V M L T H K N I V A R F  220
S I A K D P T F G N A I N P T T A I L T  240
V I P F H H G F G M M T T L G Y F T C G  260
F R V V L M H T F E E K L F L Q S L Q D  280
Y K V E S T L L V P T L M A F L A K S A  300
L V E K Y D L S H L K E I A S G G A P L  320
S K E I G E M V K K R F K L N F V R Q G  340
Y G L T E T T S A V L I T P N N D V R P  360
G S T G K I V P F H A V K V V D P T T G  380
K I L G P N E T G E L Y F K G D M I M K  400
G Y Y N N E E A T K A I I N K D G W L R  420
S G D I A Y Y D N D G H F Y I V D R L K  440
S L I K Y K G Y Q V A P A E I E G I L L  460
Q H P Y I V D A G V T G I P D E A A G E  480
L P A A G V V V Q T G K Y L N E Q I V Q  500
N F V S S Q V S T A K W L R G G V K F L  520
D E I P K G S T G K I D R K V L R Q M F  540
E K H T N G                               546
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FIG. 30

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Luc49-4G11 (SEQ ID NO:18)

M E D K N I L Y G P E P F Y P L A D 20  
G T A G E Q M F D A L S R Y A D I S G C 40  
I A L T N A H T K E N V L Y E E F L K L 60  
S C R L A E S F K K Y G L K Q N D T I A 80  
V C S E N G L Q F F L P I A S L Y L G 100  
I I A A P V S D K Y I E R E L I H S L G 120  
I V K P R I I F C S K N T F Q K V L N V 140  
K S K L K Y V E T I I I L D L N E D L G 160  
G Y Q C L N N F I S Q N S D I N L D V K 180  
K F K P Y S F N R D D Q V A L V M F S S 200  
G T T G V S K G V M L T H K N I V A R F 220  
S H A K D P T F G N A I N P T T A I L T 240  
V I P F H H G F G M M T T L G Y F T C G 260  
F R V V L M H T F E E K L F L Q S L Q D 280  
Y K V E S T L L V P T L M A F F A K S A 300  
L V E K Y D L S H L K E I A S G G A P L 320  
S K E I G E M V K K R F K L N F V R Q G 340  
Y G L T E T T S A V L I T P N N D V R P 360  
G S T G K I V P F H A V K V V D P T T G 380  
K I L G P N E T G E L Y F K G D M I M K 400  
G Y Y N N E E A T K A I I N K D G W L R 420  
S G D I A Y Y D N D G H F Y I V D R L K 440  
S L I K Y K G Y Q V A P A E I E G I L L 460  
Q H P Y I V D A G V T G I P D E A A G E 480  
L P A A G V V V Q T G K Y L N E Q I V Q 500  
N F V S S Q V S T A K W L R G G V K F L 520  
D E I P K G S T G K I D R K V L R Q M F 540  
E K H T N G 546

FIG. 31



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1uc78-0B10 (SEQ ID NO:6)

GGATCCAATG	GCAGATAAGA	ATATTTTATA	TGGGCCCGAA	CCATTTTATC	CCTTGGCTGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTGACGC	ATTATCTCGT	TATGCAGATA	TTTCGGGATG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGA	AAATGTTTTA	TATGAAGAGT	TTTTAAAATT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATGGTT	TGCAATTTTT	CCTTCCTGTA	ATTGCATCAT	TGTATCTTGG	300
AATAATTGCA	GCACCTGTTA	GTGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAA	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TTAAAATCTG	TAGAAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTCC	GATAGTAATC	TGGACGTAAA	540
AAAATTTAAA	CCATATTCTT	TTAATCGAGA	CGATCAGGTT	GCGTTGGTAA	TGTTTTCTTC	600
TGGTACAAC	GGTGTTCGGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTCACGATT	660
TTCTCTTGCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAA	CCGACGACAG	CAATTTTAA	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCOGAGTT	GTCTTAATGC	ACACGTTTGA	AGAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAAATG	GCATTTCTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACCT	AAAAGAAATT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAAAGGTG	ACGCGAGACC	1080
GGGATCAACT	GGTAAAATAG	TACCAATTTCA	CGCTGTTAAA	GTGTCTGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AACCTGGAGA	ATTGTATTTT	AAAGGCGCCA	TGATAATGAA	1200
GGGTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	GATAATGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCATTAATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	CCGGATGAAG	CCGCGGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AGATTTTGT	TCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	ACCAATGGG					1639

FIG. 32

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1uc78-0G8 (SEQ ID NO:7)

GGATCCAATG	GCAGATAAAA	ATATTTTATA	TGGGCCCGAA	CCATTTTATC	CCTTGGCTGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTTACGC	ATTATCTCGT	TATGCAGATA	TTTCAGGATG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGC	CCCTGTTTTA	TATGAAGAGT	TTTTAAAATT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATGGTT	TGCAATTTTT	CCTTCCTGTA	ATTGCATCAT	TGTATCTTGG	300
AATAATTGCA	GCACCTGTTA	GTGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCCTGG	360
TATTGTAAAA	CCACGCATAA	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TTAAAATATG	TAGAAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTCC	GATATTAATC	TTGACGTAAA	540
AAAAATTTAAA	CCAATTTCTT	TTAATCGAGA	CGATCAGGTT	GCGTTGGTAA	TGTTTTCTTC	600
TGGTACAAC	GGTGTTCGGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTCTTCCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCAACGACAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GTTCTAATGC	ACACGTTTGA	AGAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTCTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAATT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAAAxxxx	xxGTCAGACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTTC	CGCTGTTAAA	GTTGTCGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AACTGAGAGA	ATTGTATTTT	AAAGGCGACA	TGATAATGAA	1200
AGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	GATTAAGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCATTAAAT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	COGGATGAAG	CCGCGGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AAATTTTGTT	TCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGGGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	ACCAATGGG					1639

FIG. 33

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luc78-1E1 (SEQ ID NO:8)

GGATCCAATG	GCAGATAAAA	ATATTTTATA	TGGGCCCCGAA	CCATTTTATC	CCTTGGCTGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTTACGC	ATTATCTCGT	TATGCAGATA	TTTCAGGATG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGC	CCCTGTTTFA	TATGAAGAGT	TGTTAAAATT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAAATGGT	TGCAATATTT	CCTTCCTGTA	ATTGCATCAT	TGTATCTTGG	300
AATAATTGCA	GCACCTGTTA	GTGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAA	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TTAAAATATG	TAGAAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAAATTC	GATATTAATC	TTGACGTAAA	540
AAAATTTAAA	CCAATATTCT	TTAATCGAGA	CGATCAGGTT	GCCTTGGTAA	TGTTTTCTTC	600
TGGTACAACT	GGTGTTCGGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTATTGCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCAACGACAG	CAATTTTAAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GTTCTAATGC	ACACGTTTGA	AGAAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTCTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAATT	GCATCTGGTG	GCACACCTTT	960
ATCAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAAAXXXX	XXGCCAGACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTTCA	CGCTGTTAAA	GTGTGCGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AACTCTGGAGA	ATTGTATTTT	AAAGGCGCCA	TGATAATGAA	1200
GGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	AACAAAGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCATTAAAT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	CCGGATGAAG	CCGCGGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AAATTTTGTT	TCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	ACCAATGGG					1639

FIG. 34

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luc78-2B4 (SEQ ID NO:9)

GGATCCAATG	GCAGATAAAA	ATATTTTATA	TGGGCCCGAA	CCATTTTATC	CCTTGGCTGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTGACGC	ATTATCTCGT	TATGCAGATA	TTTCAGGATG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGC	CCCTGTTTTA	TATGAAGAGT	TGTTAAAATT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATGGTT	TGCAATTTTT	CCTTCCTGTA	ATTGCATCAT	TGTATCTTGG	300
ATAAATTGCA	GCACCTGTTA	GTGATAAATA	CGTTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAA	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TAAAAATATG	TAGAAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTC	GATAGTAATC	TGGACGTAAA	540
AAAATTTAAA	CCAAATTCCT	TTAATCGAGA	CGATCAGGTT	GCGTTGGTAA	TGTTTTCTTC	600
TGGTACAAC	GGTGTTCGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTCTTGA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCAACGACAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GTCTTAATGC	ACACGTTTGA	AGAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTCTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAATT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGTTTAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	COGAACxxxx	xxGCAGACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTTCA	CGCTGTTAAA	GTTGTGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AACTGGGAGA	ATTGTATTTT	AAAGGCGCCA	TGATAATGAA	1200
GGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	GATAAAGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCATTAATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	CCGGATGAAG	CCGCGGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AAATTTTGTT	TCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	ACCAATGGG					1639

FIG. 35

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luc78-0B10 (SEQ ID NO:19)

	M	A	D	K	N	I	L	Y	G	P	E	P	F	Y	P	L	A	D	20
G	T	A	G	E	Q	M	F	D	A	L	S	R	Y	A	D	I	S	G	40
I	A	L	T	N	A	H	T	K	E	N	V	L	Y	E	E	F	L	K	60
S	C	R	L	A	E	S	F	K	K	Y	G	L	K	Q	N	D	T	I	80
V	C	S	E	N	G	L	Q	F	F	L	P	V	I	A	S	L	Y	L	100
I	I	A	A	P	V	S	D	K	Y	I	E	R	E	L	I	H	S	L	120
I	V	K	P	R	I	I	F	C	S	K	N	T	F	Q	K	V	L	N	140
K	S	K	L	K	S	V	E	T	I	I	I	L	D	L	N	E	D	L	160
G	Y	Q	C	L	N	N	F	I	S	Q	N	S	D	S	N	L	D	V	180
K	F	K	P	Y	S	F	N	R	D	D	Q	V	A	L	V	M	F	S	200
G	T	T	G	V	P	K	G	V	M	L	T	H	K	N	I	V	A	R	220
S	L	A	K	D	P	T	F	G	N	A	I	N	P	T	T	A	I	L	240
V	I	P	F	H	H	G	F	G	M	M	T	T	L	G	Y	F	T	C	260
F	R	V	Y	L	M	H	T	F	G	E	K	L	F	L	Q	S	L	Q	280
Y	K	V	E	S	T	L	L	V	P	T	L	M	A	F	L	A	K	S	300
L	V	E	K	Y	D	L	S	H	L	K	E	I	A	S	G	G	A	P	320
S	K	E	I	G	E	M	V	K	K	R	F	K	L	N	F	V	R	Q	340
Y	G	L	T	E	T	T	S	A	V	L	I	T	P	K	G	D	A	R	360
G	S	T	G	K	I	V	P	F	H	A	V	K	V	V	D	P	T	T	380
K	I	L	G	P	N	E	P	G	E	L	Y	F	K	G	A	M	I	M	400
G	Y	Y	N	N	E	E	A	T	K	A	I	I	D	N	D	G	W	L	420
S	G	D	I	A	Y	Y	D	N	D	G	H	F	Y	I	V	D	R	L	440
S	L	I	K	Y	K	G	Y	Q	V	A	P	A	E	I	E	G	I	L	460
Q	H	P	Y	I	V	D	A	G	V	T	G	I	P	D	E	A	A	G	480
L	P	A	A	G	V	V	V	Q	T	G	K	Y	L	N	E	Q	I	V	500
D	F	V	S	S	Q	V	S	T	A	K	W	L	R	G	G	V	K	F	520
E	K	H	T	N	G	S	T	G	K	I	D	R	K	V	L	R	Q	M	540
																			546

FIG. 36

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Luc78-0G8 (SEQ ID NO:20)

M A D K N I L Y G P E P F Y P L A D 20  
G T A G E Q M F Y A L S R Y A D I S G C 40  
I A L T N A H T K E N V L Y E E F L K L 60  
S C R L A E S F K K Y G L K Q N D T I A 80  
V C S E N G L Q F F L P V I A S L Y L G 100  
I I A A P V S D K Y I E R E L I H S L G 120  
I V K P R I I F C S K N T F Q K V L N V 140  
K S K L K Y V E T I I I L D L N E D L G 160  
G Y Q C L N N F I S Q N S D I N L D V K 180  
K F K P Y S F N R D D Q V A L V M F S S 200  
G T T G V P K G V M L T H K N I V A R F 220  
S L A K D P T F G N A I N P T T A I L T 240  
V I P F H H G F G M M T T L G Y F T C G 260  
F R V V L M H T F E E K L F L Q S L Q D 280  
Y K V E S T L L V P T L M A F L A K S A 300  
L V E K Y D L S H L K E I A S G G A P L 320  
S K E I G E M V K K R F K L N F V R Q G 340  
Y G L T E T T S A V L I T P K X X V R P 360  
G S T G K I V P F H A V K V V D P T T G 380  
K I L G P N E P G E L Y F K G D M I M K 400  
G Y Y N N E E A T K A I I D K D G W L R 420  
S G D I A Y Y D N D G H F Y I V D R L K 440  
S L I K Y K G Y Q V A P A E I E G I L L 460  
Q H P Y I V D A G V T G I P D E A A G E 480  
L P A A G V V V Q T G K Y L N E Q I V Q 500  
N F V S S Q V S T A K W L R G G V K F L 520  
D E I P K G S T G K I D R K V L R Q M F 540  
E K H T N G 546

FIG. 37

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Luc78-1E1 (SEQ ID NO:21)

M A D K N I L Y G P E P F Y P L A D 20  
G T A G E Q M F D A L S R Y A D I P G C 40  
I A L T N A H T K E N V L Y E E F L K L 60  
S C R L A E S F K K Y G L K Q N D T I A 80  
V C S E N G L Q Y F L P V I A S L Y L G 100  
I I A A P V S D K Y I E R E L I H S L G 120  
I V K P R I I F C S K N T F Q K V L N V 140  
K S K L K Y V E T I I I L D L N E D L G 160  
G Y Q C L N N F I S Q N S D I N L D V K 180  
K F K P N S F N R D D Q V A L V M F S S 200  
G T T G V P K G V M L T H K N I V A R F 220  
S I A K D P T F G N A I N P T T A I L T 240  
V I P F H H G F G M M T T L G Y F T C G 260  
F R V V L M H T F E E K L F L Q S L Q D 280  
Y K V E S T L L V P T L M A F L A K S A 300  
L V E K Y D L S H L K E I A S G G A P L 320  
S K E I G E M V K K R F K L N F V R Q G 340  
Y G L T E T T S A V L I T P K X X A R P 360  
G S T G K I V P F H A V K V V D P T T G 380  
K I L G P N E P G E L Y F K G A M I M K 400  
G Y Y N N E E A T K A I I D K D G W L R 420  
S G D I A Y Y D N D G H F Y I V D R L K 440  
S L I K Y K G Y Q V A P A E I E G I L L 460  
Q H P Y I V D A G V T G I P D E A A G E 480  
L P A A G V V V Q T G K Y L N E Q I V Q 500  
N F V S S Q V S T A K W L R G G V K F L 520  
D E I P K G S T G K I D R K V L R Q M F 540  
E K H T N G 546

FIG. 38

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Luc78-2B4 (SEQ ID NO:22)

M A D K N I L Y G P E P F Y P L A D 20  
G T A G E Q M F D A L S R Y A D I P G C 40  
I A L T N A H T K E N V L Y E E F L K L 60  
S C R L A E S F K K Y G L K Q N D T I A 80  
V C S E N G L Q F F L P Y I A S L Y L G 100  
I I A A P V S D K Y Y E R E L I H S L G 120  
I V K P R I I F C S K N T F Q K V L N V 140  
K S K L K Y V E T I I I L D L N E D L G 160  
G Y Q C L N N F I S Q N S D S N L D V K 180  
K F K P N S F N R D D Q V A L V M F S S 200  
G T T G V P K G V M L T H K N I V A R F 220  
S L A K D P T F G N A I N P T T A I L T 240  
V I P F H H G F G M M T T L G Y F T C G 260  
F R V V L M H T F E E K L F L Q S L Q D 280  
Y K V E S T L L V P T L M A F L A K S A 300  
L V E K Y D L S H L K E I A S G G A P L 320  
S K E I G E M V K K R F K L N F V R Q G 340  
Y G L T E T T S A V L I T P K X X A R P 360  
G S T G K I V P F H A V K V V D P T T G 380  
K I L G P N E T G E L Y F K G A M I M K 400  
G Y Y N N E E A T K A I I D K D G W L R 420  
S G D I A Y Y D N D G H F Y I V D R L K 440  
S L I K Y K G Y Q V A P A E I E G I L L 460  
Q H P Y I V D A G V T G I P D E A A G E 480  
L P A A G V V V Q T G K Y L N E Q I V Q 500  
N F V S S Q V S T A K W L R G G V K F L 520  
D E I P K G S T G K I D R K V L R Q M F 540  
E K H T N G 546

FIG. 39



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luc85-4F12 (SEQ ID NO:10)

GGATCCAATG	GCAGATAAAA	ATATTTTATA	TGGGCCCGAA	CCATTTTATC	CCTTGGCTGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTACGC	ATTATCTCGT	TATGCAGATA	TTCCGGGGCTG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGC	CCCTGTTTTA	TATGAAGAGT	TTTTAAAATT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATGGTT	TGCAATTTTT	CCTTCCTGTA	ATTGCATCAT	TGTATCTTGG	300
AATAATGTG	GCACCTGTTA	ACGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAG	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TTAAAATCTG	TAGAAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTCC	GATATTAATC	TTGACGTAAA	540
AAAATTTAAA	CCAATTTCTT	TTAATCGAGA	CGATCAGGTT	GCGTTGATTA	TGTTTTCTTC	600
TGGTACAACT	GGTCTGCCGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTCTTCCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCACGACAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GTTCTAATGC	ACACGTTTGA	AGAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTCTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAATT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAAAxxxx	xxGCCAGACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTTCA	CGCTGTTAAA	GTTGTGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AACTGGGAGA	ATTGTATTTT	AAAGGCCCGA	TGATAATGAA	1200
GGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	GATAATGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCATTAATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATT	CCGGATGAAG	CCGCCGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AAATTTTGTT	TCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAG	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	ACCAATGGG					1639

FIG. 40

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Luc85-4F12 (SEQ ID NO:23)

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      M A D K N I L Y G P E P F Y P L A D   20
G T A G E Q M F D A L S R Y A D I P G C   40
I A L T N A H T K E N V L Y E E F L K L   60
S C R L A E S F K K Y G L K Q N D T I A   80
V C S E N G L Q F F L P V I A S L Y L G  100
I I V A P V N D K Y I E R E L I H S L G  120
I V K P R I I F C S K N T F Q K V L N V  140
K S K L K S V E T I I I L D L N E D L G  160
G Y Q C L N N F I S Q N S D I N L D V K  180
K F K P Y S F N R D D Q V A L I M F S S  200
G T T G L P K G V M L T H K N I V A R F  220
S L A K D P T F G N A I N P T T A I L T  240
V I P F H H G F G M M T T L G Y F T C G  260
F R V V L M H T F E E K L F L Q S L Q D  280
Y K V E S T L L V P T L M A F L A K S A  300
L V E K Y D L S H L K E I A S G G A P L  320
S K E I G E M V K K R F K L N F V R Q G  340
Y G L T E T T S A V L I T P K X X A R P  360
G S T G K I V P F H A V K V V D P T T G  380
K I L G P N E P G E L Y F K G P M I M K  400
G Y Y N N E E A T K A I I D N D G W L R  420
S G D I A Y Y D N D G H F Y I V D R L K  440
S L I K Y K G Y Q V A P A E I E G I L L  460
Q H P Y I V D A G V T G I P D E A A G E  480
L P A A G V V V Q T G K Y L N E Q I V Q  500
D F V S S Q V S T A K W L R G G V K F L  520
D E I P K G S T G K I D R K V L R Q M F  540
E K H T N G                               546

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FIG. 41

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Luc90-1B5 (SEQ ID NO:11)

GGATCCAATG	GCAGATAAGA	ATATTTTATA	TGGGCCCGAA	CCATTTTATC	CCTTGGAAAGA	60
TGGGACCGCT	GGAGAACAGA	TGTTTGACGC	ATTATCTCGT	TATGCAGATA	TTCCGGGGCTG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGA	AAATGTTTTA	TATGAAGAGT	TTCTGAAACT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATGGTC	TGCAATTTTT	CCTTCCTGTA	ATTGCATCAT	TGTATCTTGG	300
AATAATGTGT	GCACCTGTTA	ACGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAG	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAAATCTAA	TTAAAATCTA	TTGAAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTCC	GATAGTAATC	TGGACGTAAA	540
AAAAATTTAAA	CCATATTCTT	TTAATCGAGA	CGATCAGGTT	GCGTGGATTA	TGTTTTCTTC	600
TGGTACAACCT	GGTCTGCCGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTCTTGCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCCACGACAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GTCTAATGTC	ACACGTTTGA	AGAAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTAAC	AACATTAATG	GCATTTCTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAATT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAACTTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAAAGGTG	ACGCCAACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTTCA	CGCTGTTAAA	GTTGTGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AACTGGGAGA	ATTGTATTTT	AAAGGCCCGA	TGATAATGAA	1200
GGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	GATAATGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCACTGATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	CCGGATGAAG	CCGCCGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AGATTATGTT	GCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	ACCAATGGG					1639

FIG. 42

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Luc90-1B5 (SEQ ID NO:24)

	M	A	D	K	N	I	L	Y	G	P	E	P	F	Y	P	L	E	D	20		
G	T	A	G	E	Q	M	F	D	A	L	S	R	Y	A	D	I	P	G	C	40	
I	A	L	T	N	A	H	T	K	E	N	V	L	Y	E	E	F	L	K	L	60	
S	C	R	L	A	E	S	F	K	K	Y	G	L	K	Q	N	D	T	I	A	80	
V	C	S	E	N	G	L	Q	F	F	L	P	V	I	A	S	L	Y	L	G	100	
I	I	V	A	P	V	N	D	K	Y	I	E	R	E	L	I	H	S	L	G	120	
I	V	K	P	R	I	V	F	C	S	K	N	T	F	Q	K	V	L	N	V	140	
K	S	K	L	K	E	I	E	T	I	I	I	L	D	F	L	N	E	D	L	G	160
G	Y	Q	C	L	N	N	F	I	S	Q	N	S	D	S	N	L	D	V	K	180	
K	F	K	P	Y	S	F	N	R	D	D	Q	V	A	L	I	M	F	S	S	200	
G	T	T	G	L	P	K	G	V	M	L	T	H	K	N	I	V	A	R	F	220	
S	L	A	K	D	P	T	F	G	N	A	I	N	P	T	T	A	I	L	T	240	
V	I	P	F	H	H	G	F	G	M	M	T	T	L	G	Y	F	T	C	G	260	
F	R	V	Y	L	M	H	T	F	E	E	K	L	F	L	Q	S	L	Q	D	280	
Y	K	V	E	S	T	L	L	V	P	T	L	M	A	F	L	A	K	S	A	300	
L	V	E	K	Y	D	L	S	H	L	K	E	I	A	S	G	G	A	P	L	320	
S	K	E	I	G	E	M	V	K	K	R	F	K	L	N	F	V	R	Q	G	340	
Y	G	L	T	E	T	T	S	A	V	L	I	T	P	K	G	D	A	K	P	360	
G	S	T	G	K	I	V	P	F	H	A	V	K	V	V	D	P	T	T	G	380	
K	I	L	G	P	N	E	P	G	E	L	Y	F	K	G	P	M	I	M	K	400	
Q	Y	Y	N	N	E	E	A	T	K	A	I	I	D	N	D	G	W	L	R	420	
S	G	D	I	A	Y	Y	D	N	D	G	H	F	Y	I	V	D	R	L	K	440	
S	L	I	K	Y	K	G	Y	Q	V	A	P	A	E	I	E	G	I	L	L	460	
Q	H	P	Y	I	V	D	A	G	V	T	G	I	P	D	E	A	A	G	E	480	
L	P	A	A	G	V	V	V	Q	T	G	K	Y	L	N	E	Q	I	V	Q	500	
D	Y	V	A	S	Q	V	S	T	A	K	W	L	R	G	G	V	K	F	L	520	
D	E	I	P	K	G	S	T	G	K	I	D	R	K	V	L	R	Q	M	F	540	
E	K	H	T	N	G															546	

FIG. 43

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1ucPpe2 [T249M] (SEQ ID NO:12)

GGATCCAATG	GAAGATAAAA	ATATTTTATA	TGGACCTGAA	CCATTTTATC	CCTTGGCTGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTTACGC	ATTATCTCGT	TATGCAGATA	TTTCAGGATG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGA	AAATGTTTTA	TATGAAGAGT	TTTTAAAATT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATGGTT	TGCAATTTTT	CCTTCCTTTA	ATTGCATCAT	TGTATCTTGG	300
AATAATTGCA	GCACCTGTTA	GTGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAA	TTTTTTGTTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TTAAAAATATG	TAGAAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTCC	GATATTAAATC	TTGACGTAAA	540
AAAATTTAAA	CCAAATTCCTT	TTAATCGAGA	CGATCAGGTT	GCGTTGGTAA	TGTTTTCTTC	600
TGGTACAAC	GGTGTTCGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTCATTC	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCAACGACAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GCTCTAATGC	ACACGTTTGA	AGAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTTTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAAAT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGGACACTG	ACGTCAGACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTTCA	CGCTGTAAAA	GTTGTCGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AAACTGGAGA	ATTGTATTTT	AAAGGCGACA	TGATAATGAA	1200
AAGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	AACAAAGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCATTAATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTG	ATGCCGGCGT	TACTGGTATA	CCGGATGAAG	CCGCGGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AAATTTTGTT	TCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
TGAAAAACAC	AAATCTAAGC	TG				1642

FIG. 44

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LucPpe2 [T249M] (SEQ ID NO:25)

```
      M E D K N I L Y G P E P F Y P L A D 20
G T A G E Q M F Y A L S R Y A D I S G C 40
I A L T N A H T K E N V L Y E E F L K L 60
S C R L A E S F K K Y G L K Q N D T I A 80
V C S E N G L Q F F L P L I A S L Y L G 100
I I A A P V S D K Y I E R E L I H S L G 120
I V K P R I I F C S K N T F Q K V L N V 140
K S K L K Y V E T I I I L D L N E D L G 160
G Y Q C L N N F I S Q N S D I N L D V K 180
K F K P N S F N R D D Q V A L V M F S S 200
G T T G V S K G V M L T H K N I V A R F 220
S H C K D P T F G N A I N P T T A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V A L M H T F E E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F F A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P D T D V R P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E T G E L Y F K G D M I M K 400
G Y Y N N E E A T K A I I N K D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
N F V S S Q V S T A K W L R G G V K F L 520
D E I P K G S T G K I D R K V L R Q M F 540
E K H K S K L 547
```

FIG. 45

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LucPpL 81-6G1 (SEQ ID NO:26)

M M K R E K N V I Y G P E P L H P L E D 20  
L T A G E M L F R A L R K H S H L P Q A 40  
L V D V V G D E S L S Y K E F F E A T V 60  
L L A Q S L H N C G Y K M N D V V S I C 80  
A E N N T R F F I P V I A A W Y I G M I 100  
V A P V N E S Y I P D E L C K V M G I S 120  
K P Q I V F T T K N I L N K V L E V Q S 140  
R T N F I K R I I I L D T V E N I H G C 160  
E S L P N G I S R Y S D G N I A N F K P 180  
L H E D P V E Q V A A I L C S S G T T G 200  
L P K G V M Q T H Q N I C V R L I H A L 220  
D P R A G T Q L I P G V T V L V Y L P F 240  
F H A F G F S I T L G Y F M V G L R V I 260  
M E R R F D Q E A F L K A I Q D Y E V R 280  
S V I N V P S V I L F L S K S P L V D K 300  
Y D L S S L R E L C C G A A P L A K E V 320  
A E V A A K R L N L P G I R C G F G L T 340  
E S T S A N I H S L R D E F K S G S L G 360  
R V T P L M A A K I A D R E T G K A L G 380  
P N Q V G E L C I K G P M V S K G Y V N 400  
N V E A T K E A I D D D G W L H S G D F 420  
G Y Y D E D E H F Y V V D R Y K E L I K 440  
Y K G S Q V A P A E L E E I L L K N P C 460  
I R D V A V V G I P D L E A G E L P S A 480  
F V V K Q P G K E I T A K E V Y D Y L A 500  
E R V S H T K Y L R G G V R F V D S I P 520  
R N V T G K I T R K E L L K Q L L E K A 540  
G G 542

FIG. 46

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LucPpl 81-6G1

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ATGATGAAGC GAGAGAAAAA TGTATATAT GGACCCGAAC CCTACACCC CTTGGAAGAC
TTAACAGCTG GAGAAATGCT CTTCGGTGCC CTTCGAAAAC ATTCTCATTT ACCGCAGGCT
TTAGTAGATG TGTTGGCGA CGAATCGCTT TCCTATAAAG AGTTTTTTGA AGCGACAGTC
CTCCTAGCGC AAAGTCTCCA CAATTGTGGA TACAAGATGA ATGATGTAGT GTCGATCTGC
GCCGAGAATA ATACAAGATT TTTATTCCTT GTTATTGCAG CTTGGTATAT TGGTATGATT
GTAGCACCTG TTAATGAAAAG TTACATCCCA GATGAACTCT GTAAGGTGAT GGGTATATCG
AAACCACAAA TAGTTTTTAC GACAAAGAAC ATTTTAAATA AGGTATTGGA GGTACAGAGC
AGAACTAATT TCATAAAAAG GATCATCATA CTTGATACTG TAGAAAACAT ACACGGTTGT
GAAAGTCTTC CCAATTTTAT TTCTCGTTAT TCGGATGGAA ATATTGCCAA CTTCAAACCT
TTACATTTCG ATCCTGTTGA GCAAGTGGCA GCTATCTTAT GTTCGTCAGG CACTACTGGA
TTACCGAAAAG GTGTAATGCA AACTCACCAA AATATTTGTG TCCGACTTAT ACATGCTTTA
GACCCCGAGG CAGGAACGCA ACTTATTCCT GGTGTGACAG TCCTAGTATA TCTGCCTTTT
TTCCATGCTT TTGGGTTCTC TATAAGCTTG GGATACTTCA TGGTGGGTCT TCGTGTATC
ATGTTAGAC GATTTGATCA AGAAGCATTT CTAAAAGCTA TTCAGGATTA TGAAGTTCGA
AGTGTAAATTA ACGTTCCATC AGTAATATTG TTCTTATCGA AAAGTCCTTT GGTGACAAA
TACGATTTAT CAAGTTTAAG GGAATTGTGT TCGGTGCGG CACCATTAGC AAAAGAAGTT
GCTGAGGTTG CAGCAAAACG ATTAAACTTG CCAGGAATTC GCTGTGGATT TGGTTTGACA
GAATCTACTT CAGCTAATAT ACACAGTCTT AGGGATGAAT TTAAATCAGG ATCACTTGA
AGAGTTACTC CTTTAATGGC AGCTAAAATA GCAGATAGGG AAACCTGGTAA AGCATTGGGA
CCAAATCAAG TTGGTGAATT ATGCATTAAA GGTCCCATGG TATCGAAAGG TTACGTGAAC
AATGTAGAAG CTACCAAAGA AGCTATTGAT GATGATGGTT GGCTTCACTC TGGAGACTTT
GGATACTATG ATGAGGATGA GCATTTCAT GTGGTGGACC GTTACAAGGA ATTGATTAAA
TATAAGGGCT CTCAGGTAGC ACCTGCAGAA CTAGAAGAGA TTTTATTGAA AAATCCATGT
ATCAGAGATG TTGCTGTGCT TGGTATTCCT GATCTAGAAG CTGGAGAACT GCCATCTGCG
TTTGTGGTTA AACAGCCCGG AAAGGAGATT ACAGCTAAAG AAGTGTACGA TTATCTTGCC
GAGAGGGTCT CCCATACAAA GTATTTGCGT GGAGGGGTTT GATTCTGTGA TAGCATACCA
AGGAATGTTA CAGGTAAAAT TACAAGAAAG GAACTTCTGA AGCAGTTGCT GGAGAAGGCG
GGAGGT

```

FIG. 47



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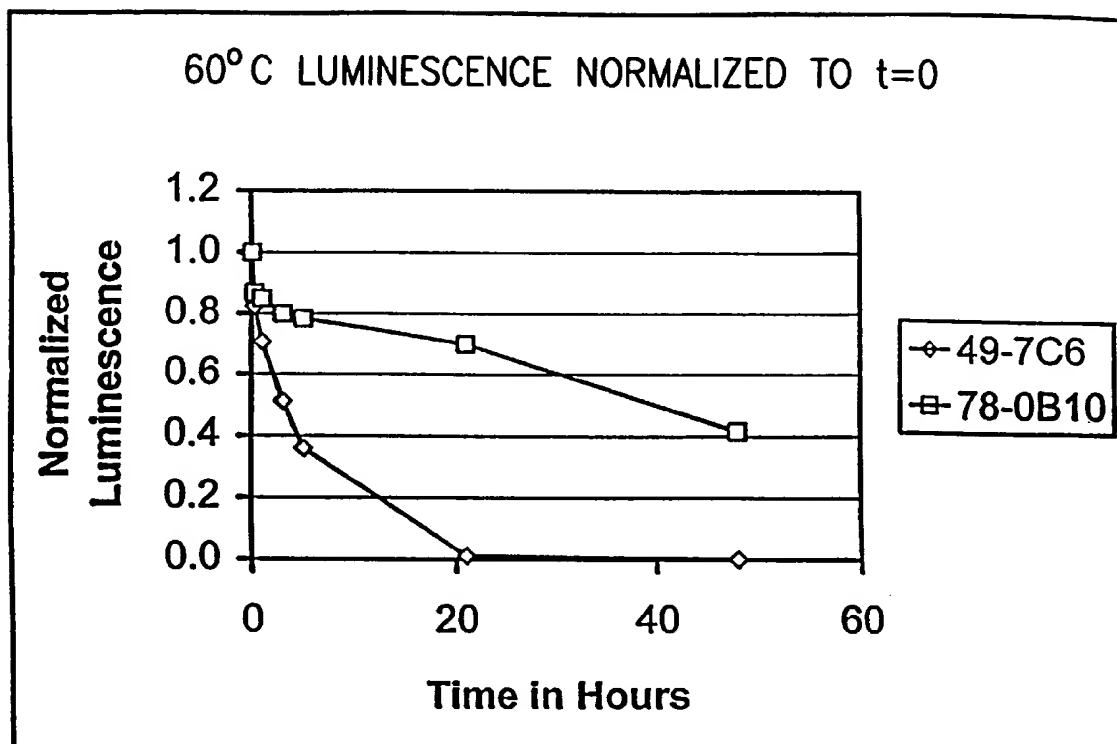


FIG. 48

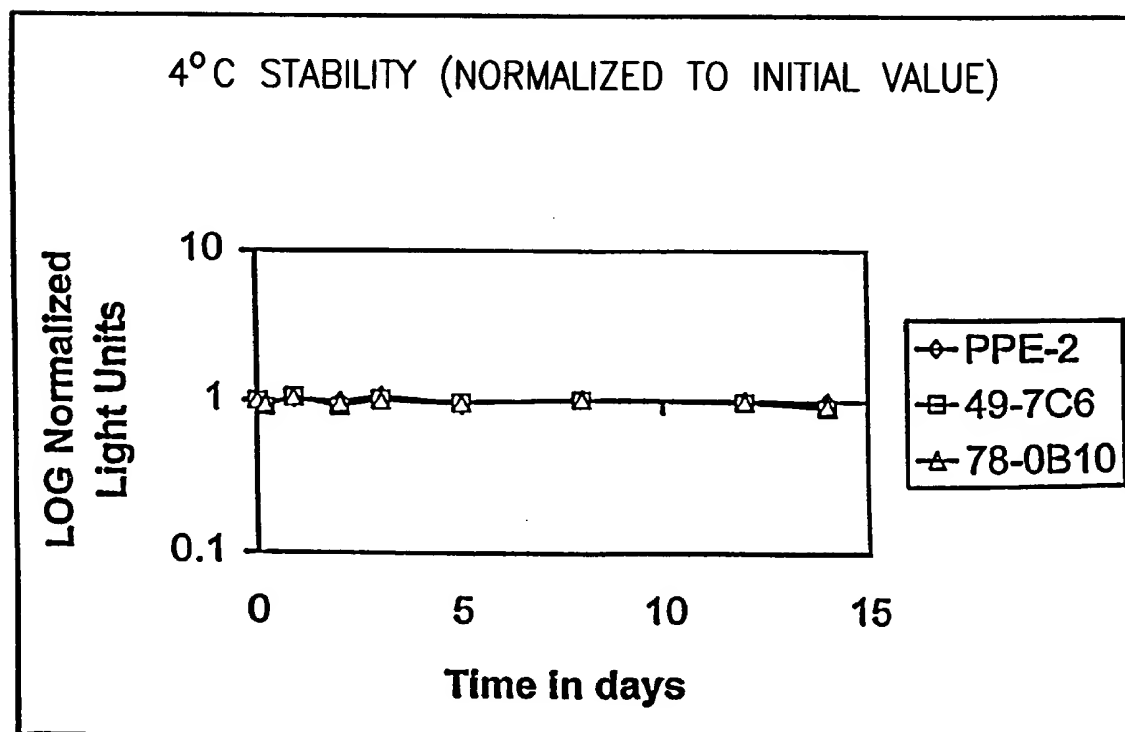


FIG. 49

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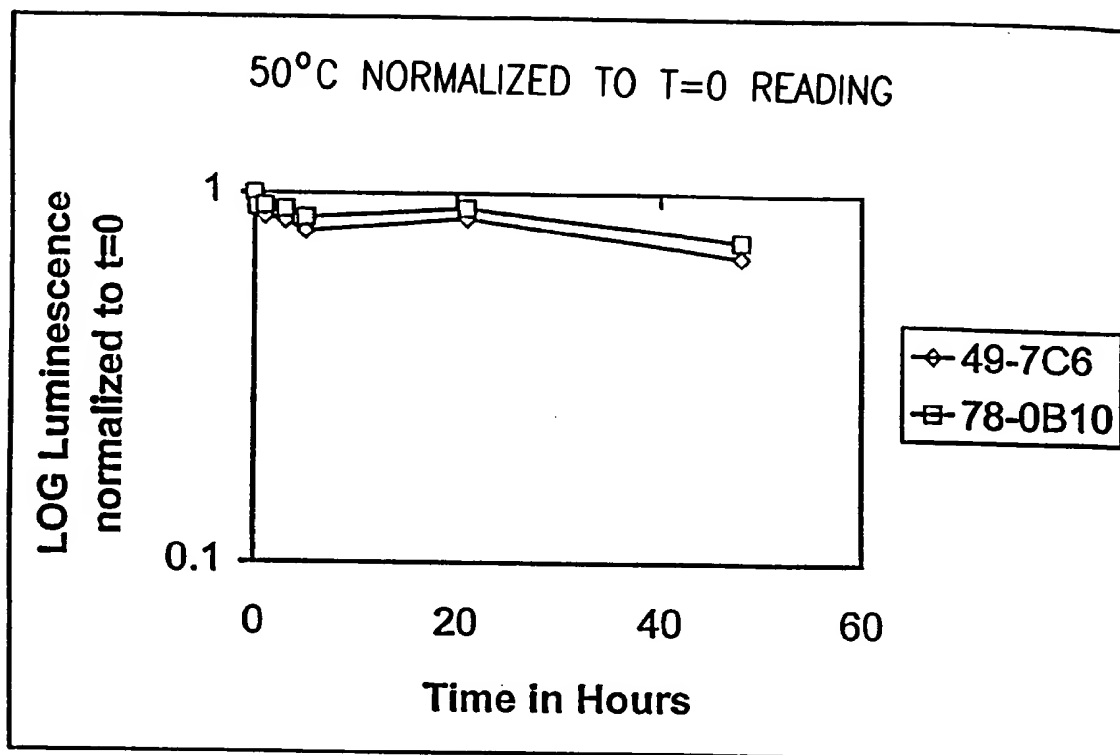


FIG. 50

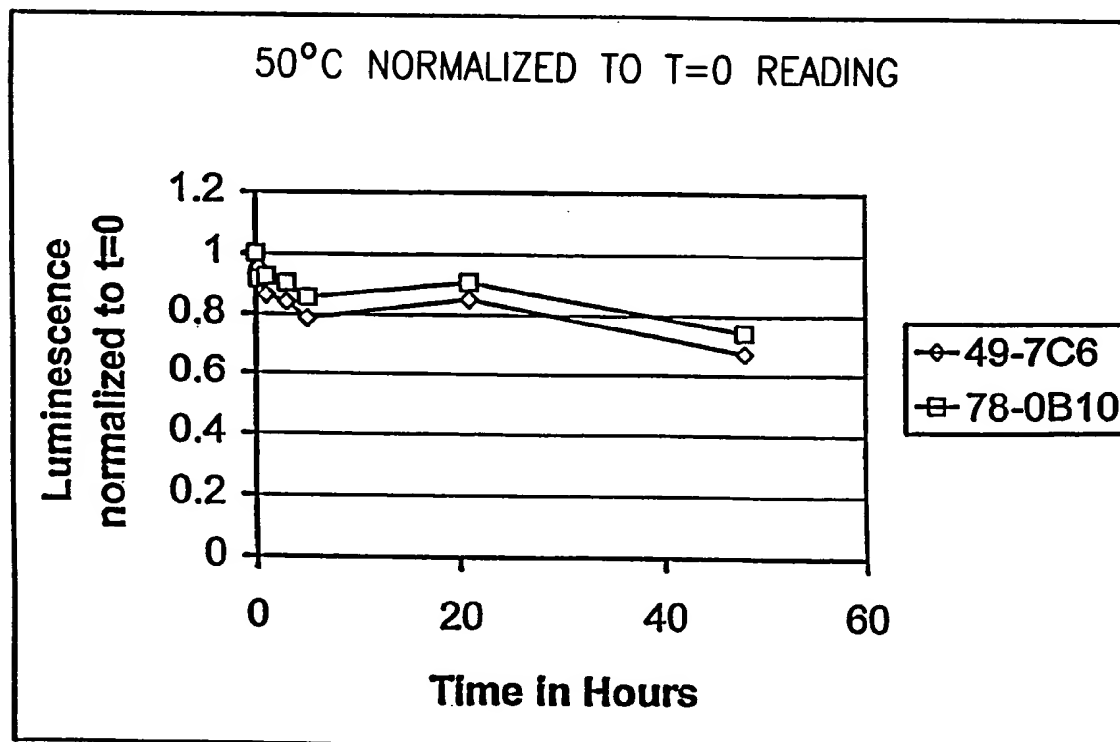


FIG. 51

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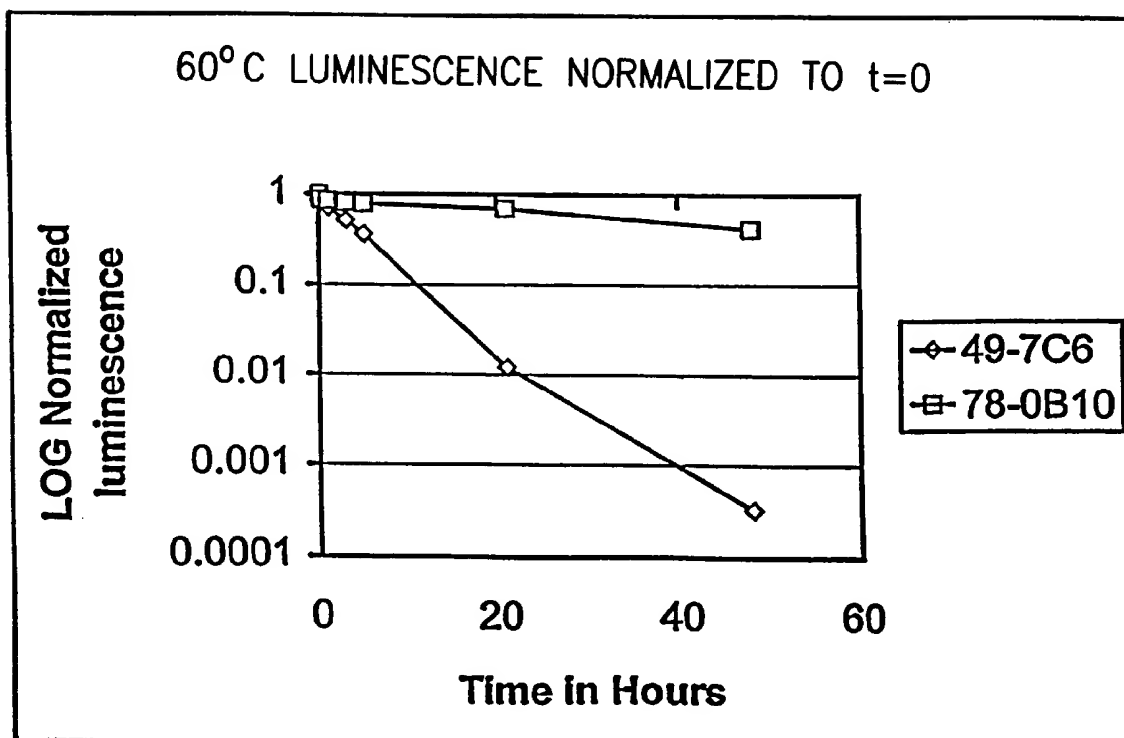


FIG. 52

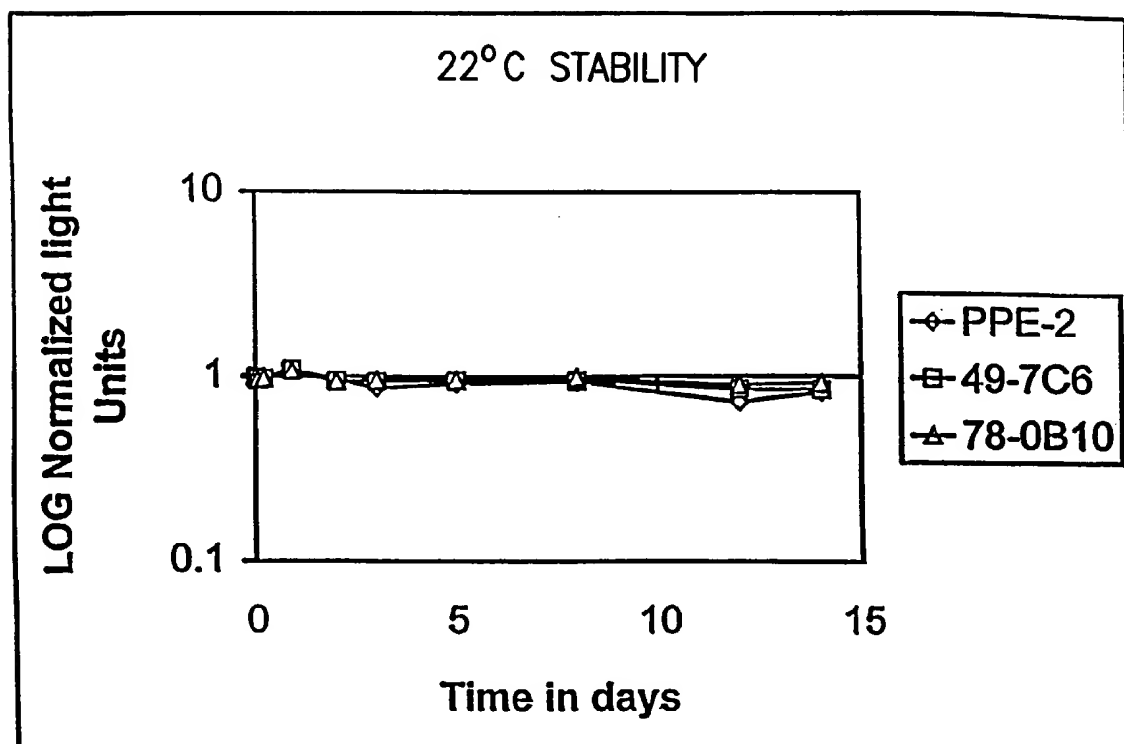


FIG. 53

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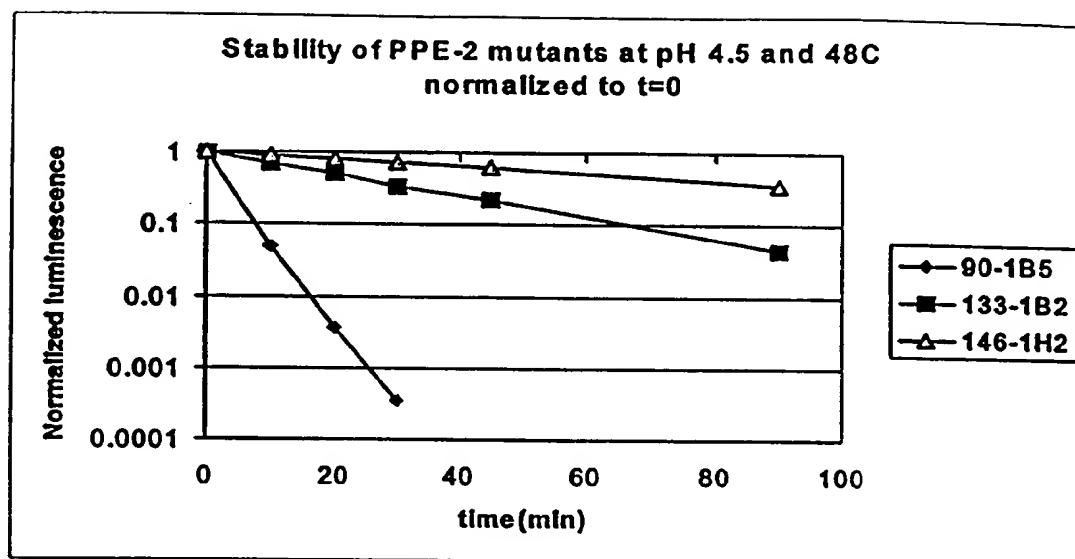


FIG. 54A

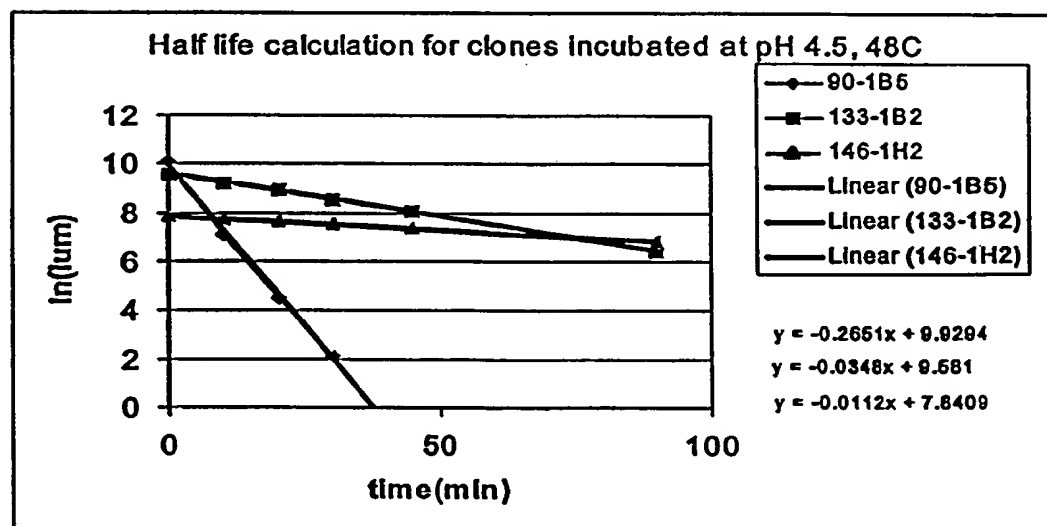


FIG. 54B

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Luc133-1B2 (SEQ ID NO:42)

<u>A</u> GATCCAATG	G <u>C</u> AGATAAGA	ATATTTTATA	TGGG <u>C</u> CCGAA	CCATTTTATC	CCTTGG <u>A</u> AGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTGACGC	ATTATCTCGT	TATGCAGATA	TT <u>C</u> CGGGCTG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGA	AAATGTTTTA	TATGAAGAGT	TTCTGAAACT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATAGTC	TGCAATTTTT	CCTTCCTGTA	ATTGCATCAT	TGTATCTTGG	300
AATAATTGTG	GCACCTGTTA	<u>A</u> CGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAG	TTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TTAAAATCTA	TTGAAACTAT	TATTATATTA	GACTTAAATG	ATGACTTAGG	480
AGGTTATCAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTC	GATAGTAATC	TGGACGTAAA	540
AAAATTTAAA	CCATATTCTT	TTAATCGAGA	CGATCAGGTT	GCGTTGATTA	TGTTTTCTTC	600
TGGTACAAC	GGTCTGCGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTATTGCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCACGTCAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GTTCTAATGC	ACACGTTTGA	AGAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTCTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAATT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAAAGGTG	ACGCCAACC	1080
GGGATCAACT	GGTAAAATAG	TACCATTICA	CGCTGTTAAA	GTTGTCGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAATG	AACTGGGAGA	ATTGTATTTT	AAAGGCCCGA	TGATAATGAA	1200
GGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	GATAATGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCAGTGATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	CCGGATGAAG	CCGCGGGCGA	1440
GCTTCCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AGATTATGTT	GCCAGTCAAG	TTTCAACAGC	CAAATGGCTA	CGTGGTGGGG	TGATATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
AGAAAAACAC	ACCAATGGG					1639

FIG. 55

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Luc146-1H2 (SEQ ID NO:43)

GGATCCAATG	GCAGATAAGA	ATATTTTATA	TGGGCCCGAA	CCATTTTATC	CCTTGGGAGA	60
TGGGACGGCT	GGAGAACAGA	TGTTTGACGC	ATTATCTCGT	TATGCAGCTA	TTCCGGGGCTG	120
CATAGCATTG	ACAAATGCTC	ATACAAAAGA	AAATGTTTTA	TATGAAGAGT	TTCTGAAACT	180
GTCGTGTCGT	TTAGCGGAAA	GTTTTAAAAA	GTATGGATTA	AAACAAAACG	ACACAATAGC	240
GGTGTGTAGC	GAAAATAGTC	TGCAATTTTT	CCTTCCTGTA	ATTGCATCAT	TGTATCTTGG	300
AATAATTGTG	GCACCTGTTA	ACGATAAATA	CATTGAACGT	GAATTAATAC	ACAGTCTTGG	360
TATTGTAAAA	CCACGCATAG	TTTTTTTGCTC	CAAGAATACT	TTTCAAAAAG	TACTGAATGT	420
AAAATCTAAA	TTAAAATCTA	TTGAAACTAT	TATTATATTA	GACTTAAATG	AAGACTTAGG	480
AGGTATACAA	TGCCTCAACA	ACTTTATTTT	TCAAAATTCC	GATAGTAATC	TGGACGTAAA	540
AAAATTTAAA	CCCTATTTCT	TTAATCGAGA	CGATCAGGTT	GCGTCCGATT	TGTTTTCTTC	600
TGGTACAAC	GGTCTGCCGA	AGGGAGTCAT	GCTAACTCAC	AAGAATATTG	TTGCACGATT	660
TTCTATTGCA	AAAGATCCTA	CTTTTGGTAA	CGCAATTAAT	CCACCGTCAG	CAATTTTAAC	720
GGTAATACCT	TTCCACCATG	GTTTTGGTAT	GATGACCACA	TTAGGATACT	TTACTTGTGG	780
ATTCCGAGTT	GTTCTAATGC	ACACGTTTGA	AGAAAACTA	TTTCTACAAT	CATTACAAGA	840
TTATAAAGTG	GAAAGTACTT	TACTTGTACC	AACATTAATG	GCATTTCTTG	CAAAAAGTGC	900
ATTAGTTGAA	AAGTACGATT	TATCGCACTT	AAAAGAAATT	GCATCTGGTG	GCGCACCTTT	960
ATCAAAAGAA	ATTGGGGAGA	TGGTGAAAAA	ACGGTTTAAA	TTAAACTTTG	TCAGGCAAGG	1020
GTATGGATTA	ACAGAAACCA	CTTCGGCTGT	TTTAATTACA	CCGAAAGGTG	ACGCCAAGCC	1080
GGGATCAACT	GGTAAAATAG	TACCATTACA	CGCTGTTAAA	GTTGTCGATC	CTACAACAGG	1140
AAAAATTTTG	GGGCCAAATG	AACTGGGAGA	ATTGTATTTT	AAAGGCCCGA	TGATAATGAA	1200
GGGTTATTAT	AATAATGAAG	AAGCTACTAA	AGCAATTATT	GATAATGACG	GATGGTTGCG	1260
CTCTGGTGAT	ATTGCTTATT	ATGACAATGA	TGGCCATTTT	TATATTGTGG	ACAGGCTGAA	1320
GTCACGTGATT	AAATATAAAG	GTTATCAGGT	TGCACCTGCT	GAAATTGAGG	GAATACTCTT	1380
ACAACATCCG	TATATTGTTG	ATGCCGGCGT	TACTGGTATA	CCGGATGAAG	CCGCGGGCGA	1440
GCTTCAGCT	GCAGGTGTTG	TAGTACAGAC	TGGAAAATAT	CTAAACGAAC	AAATCGTACA	1500
AGATTATGTT	GCCAGTCAAG	TTTCAACAGC	CAATGGCTA	CGTGGTGGGG	TGAAATTTTT	1560
GGATGAAATT	CCCAAAGGAT	CAACTGGAAA	AATTGACAGA	AAAGTGTTAA	GACAAATGTT	1620
AGAAAAACAC	ACCAATGGG					1639

FIG. 56

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Luc133-1B2 (SEQ ID NO:44)

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      M A D K N I L Y G P E P F Y P L E D 20
G T A G E Q M F D A L S R Y A D I E G C 40
I A L T N A H T K E N V L Y E E F L K L 60
S C R L A E S F K K Y G L K Q N D T I A 80
V C S E N S L Q F F L P Y I A S L Y L G 100
I I V A P V N D K Y I E R E L I H S L G 120
I V K P R I V F C S K N T F Q K V L N V 140
K S K L K S I E T I I I L D L N D D L G 160
G Y Q C L N N F I S Q N S D S N L D V K 180
K F K P Y S F N R D D Q V A L I M F S S 200
G T T G L P K G V M L T H K N I V A R F 220
S I A K D P T F G N A I N P T S A I L T 240
V I P F H H G F G M M T T L G Y F T C G 260
F R V Y L M H T F F E K L F L Q S L Q D 280
Y K V E S T L L V P T L M A F L A K S A 300
L V E K Y D L S H L K E I A S G G A P L 320
S K E I G E M V K K R F K L N F V R Q G 340
Y G L T E T T S A V L I T P K G D A K P 360
G S T G K I V P F H A V K V V D P T T G 380
K I L G P N E P G E L Y F K G P M I M K 400
G Y Y N N E E A T K A I I D N D G W L R 420
S G D I A Y Y D N D G H F Y I V D R L K 440
S L I K Y K G Y Q V A P A E I E G I L L 460
Q H P Y I V D A G V T G I P D E A A G E 480
L P A A G V V V Q T G K Y L N E Q I V Q 500
D Y V A S Q V S T A K W L R G G V I F L 520
D E I P K G S T G K I D R K V L R Q M L 540
E K H T N G

```

FIG. 57

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Luc146-1H2 (SEQ ID NO:45)

		M	A	D	K	N	I	L	Y	G	P	E	P	F	Y	P	L	E	D	20
G	T	A	G	E	Q	M	F	D	A	L	S	R	Y	A	A	I	P	G	C	40
I	A	L	T	N	A	H	T	K	E	N	V	L	Y	E	E	F	L	K	L	60
S	C	R	L	A	E	S	F	K	K	Y	G	L	K	Q	N	D	T	I	A	80
V	C	S	E	N	S	L	Q	F	F	L	P	V	I	A	S	L	Y	L	G	100
I	I	V	A	P	V	N	D	K	Y	I	E	R	E	L	I	H	S	L	G	120
K	S	K	L	K	S	I	E	F	C	S	K	N	T	F	Q	K	V	L	N	140
G	Y	Q	C	L	N	N	F	I	S	D	I	L	D	L	N	E	D	L	G	160
K	F	K	P	Y	S	F	N	R	D	Q	N	S	V	A	S	I	M	D	V	180
G	T	T	G	L	P	K	G	V	M	L	T	H	K	N	I	V	A	R	F	200
S	I	A	K	D	P	T	F	G	N	A	I	N	P	T	S	A	I	L	T	220
V	I	P	F	H	H	G	F	G	M	M	T	T	L	G	Y	F	T	C	G	240
F	R	V	Y	L	M	H	T	F	E	E	K	L	F	L	Q	S	L	Q	D	260
Y	K	V	E	S	T	L	L	V	P	T	L	M	A	F	L	A	K	S	A	280
L	V	E	K	Y	D	L	S	H	L	K	E	I	A	S	G	G	A	P	L	300
S	K	E	I	G	E	M	V	K	K	R	F	K	L	N	F	V	R	Q	G	320
Y	G	L	T	E	T	S	A	V	L	I	T	P	K	G	D	D	A	K	P	340
G	S	T	G	K	I	V	P	L	H	A	V	K	V	V	D	P	T	T	G	360
K	I	L	G	P	N	E	P	G	E	L	Y	F	K	G	P	M	I	M	K	380
Q	Y	Y	N	N	E	E	A	T	K	A	I	I	D	N	D	G	W	L	R	400
S	G	D	I	A	Y	Y	D	N	D	G	H	F	Y	I	V	D	R	L	K	420
S	L	I	K	Y	K	G	Y	Q	V	A	P	A	E	I	E	G	I	L	L	440
Q	H	P	Y	I	V	D	A	G	V	T	G	I	P	D	E	A	A	G	E	460
L	P	A	A	G	V	V	V	Q	T	G	K	W	L	R	G	V	K	F	L	480
D	Y	V	A	S	Q	V	S	T	G	K	I	D	R	K	V	L	R	Q	M	500
D	E	I	P	K	G	S	T	G	K	I	D	R	K	V	L	R	Q	M	L	520
E	K	H	T	N	G															546

FIG. 58



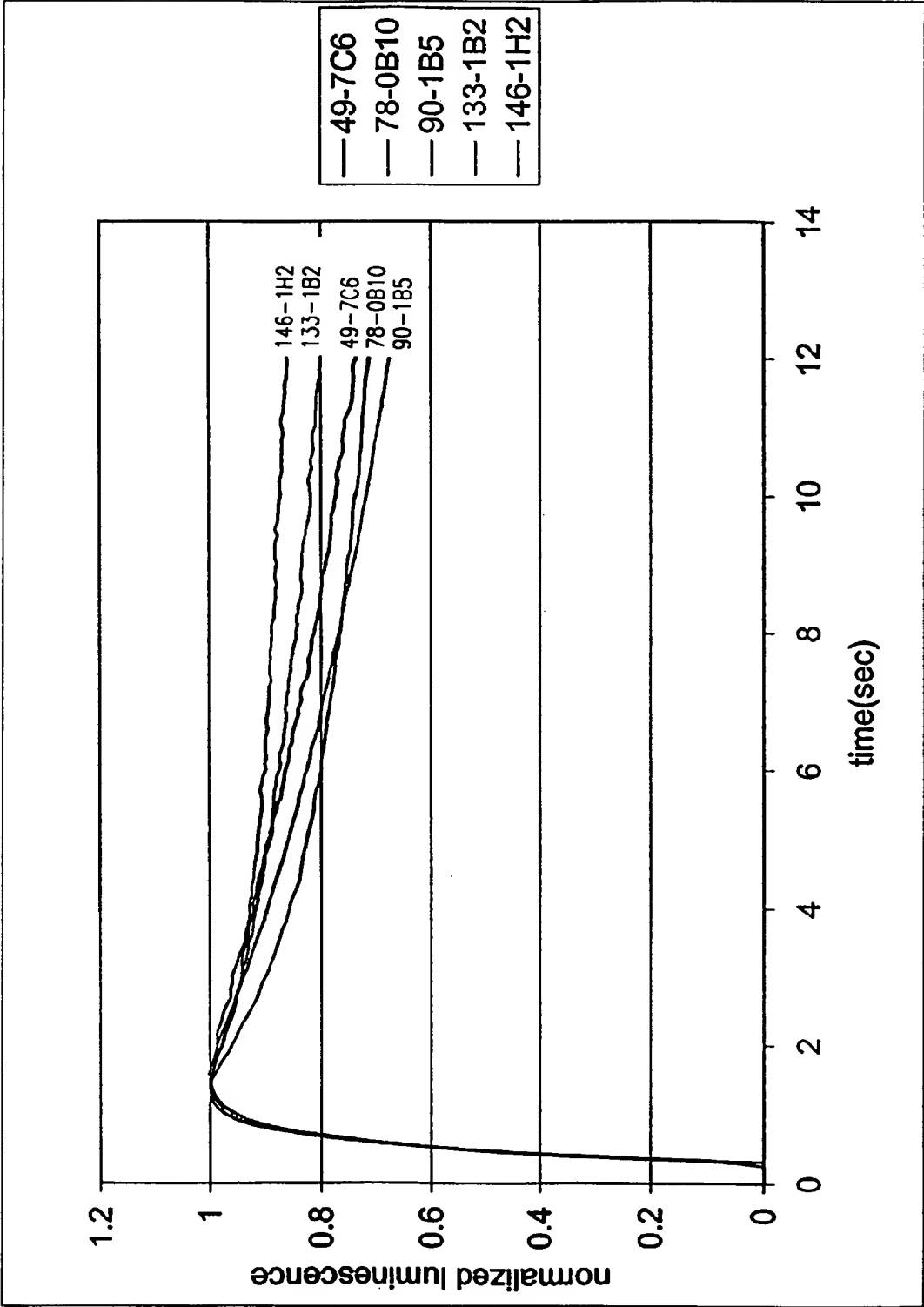


FIG. 59

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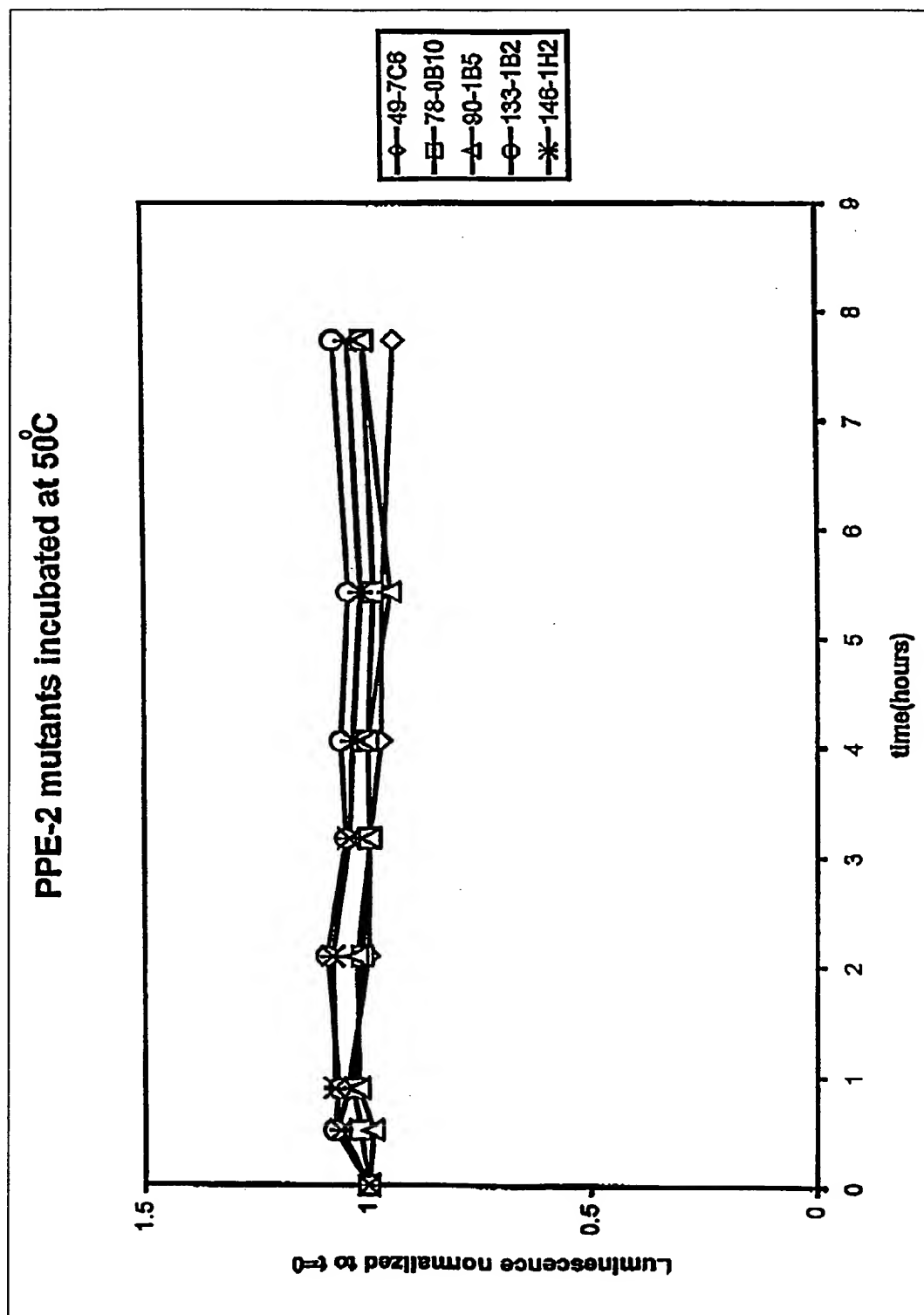


FIG. 60

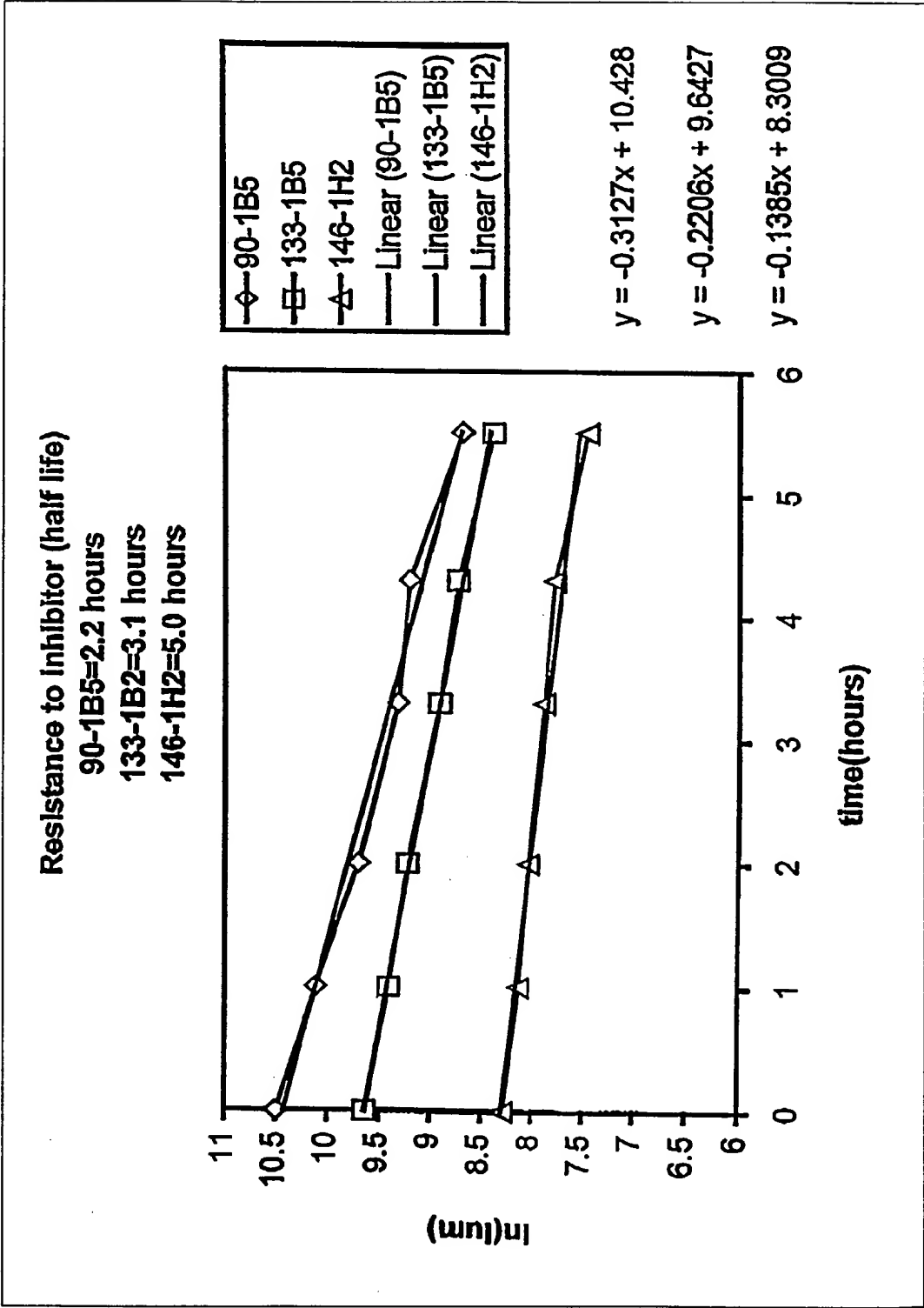


FIG. 61

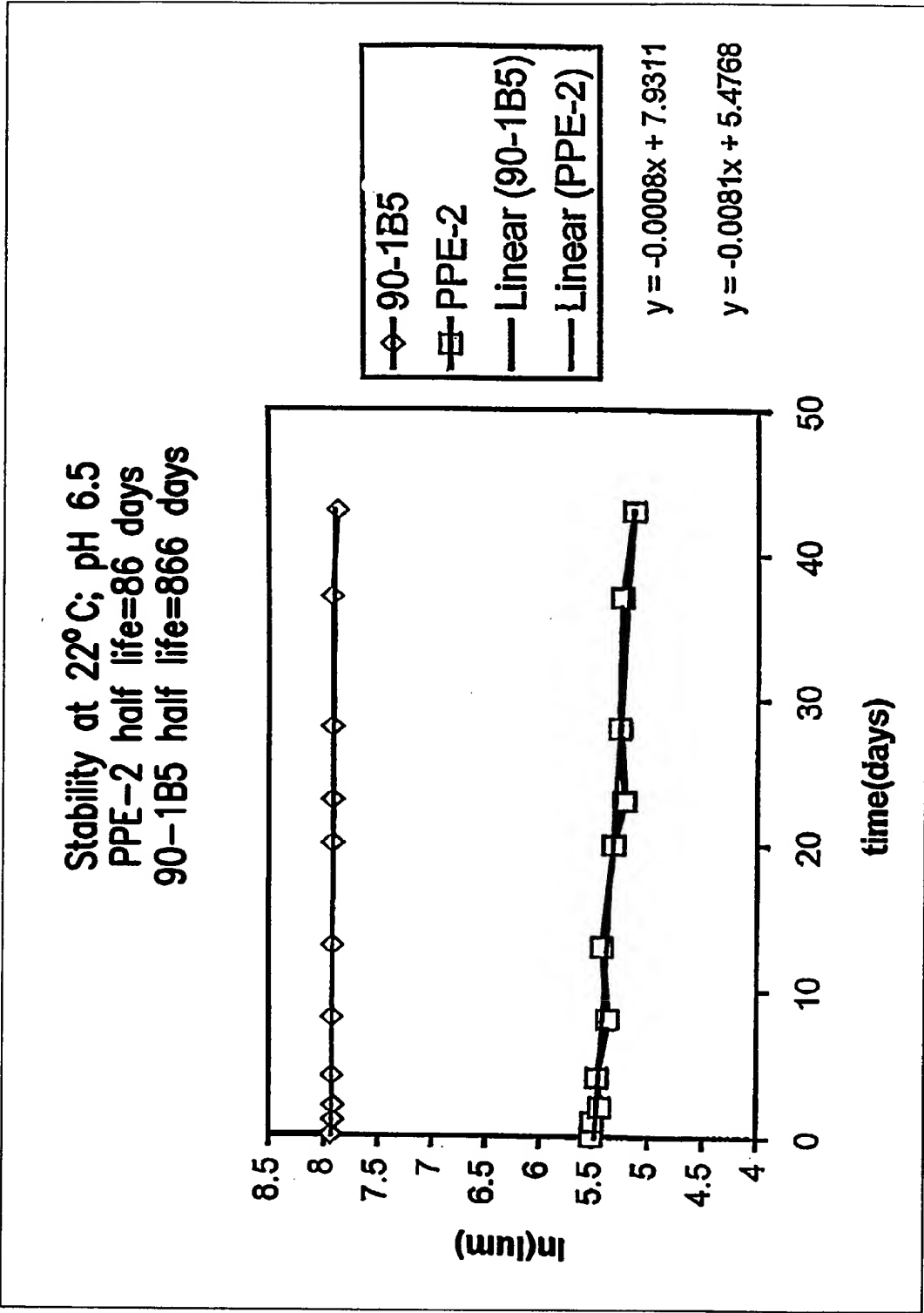


FIG. 62

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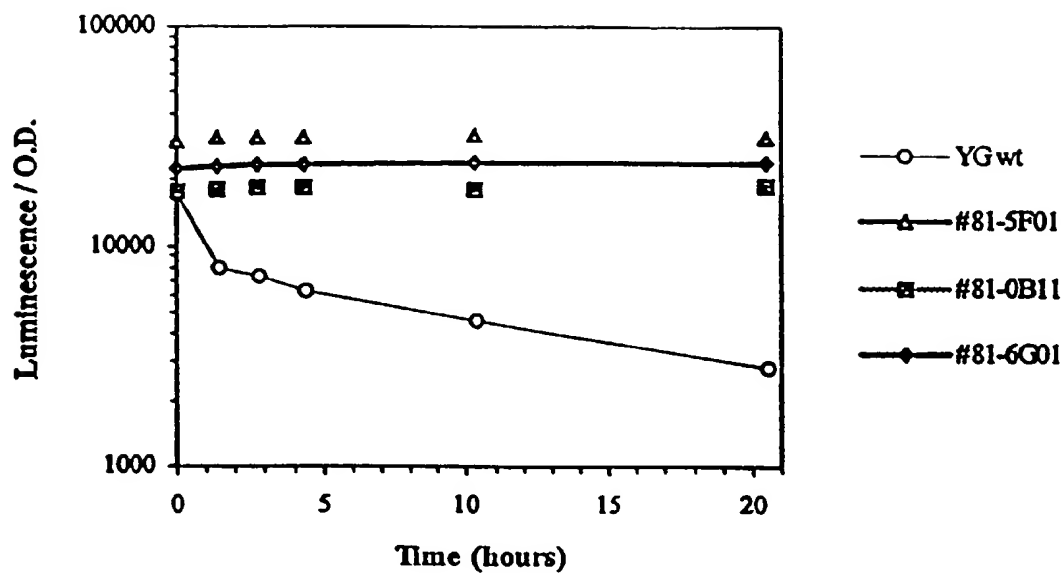


FIG. 63

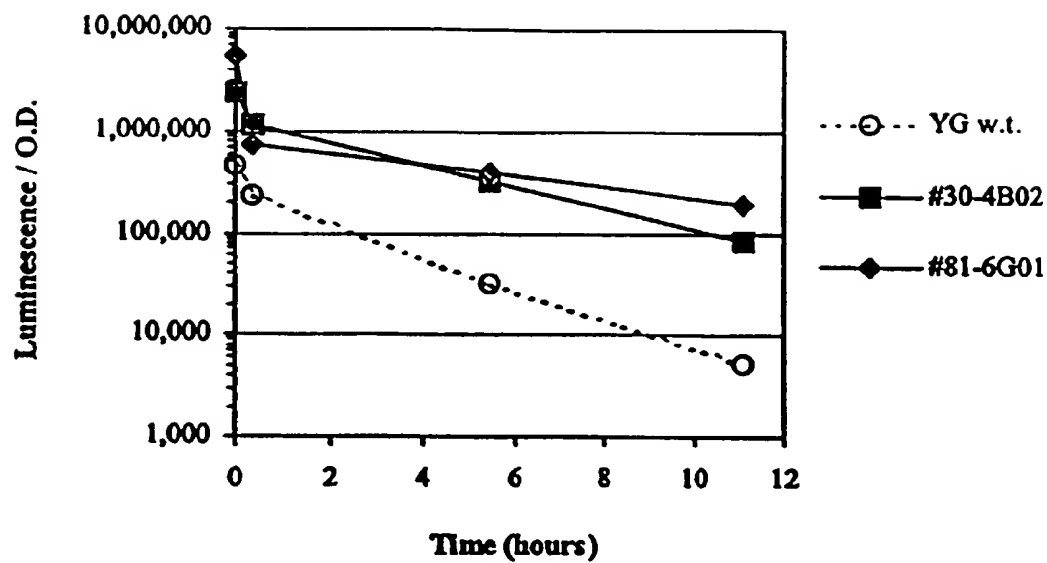


FIG. 64

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LucPpl81-0B11

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GCAGGCTTTA GTAGATGTGG TTGGCGACGA ATCGCTTTCC TATAAAGAGT TTTTGAAGC 180
GACAGTCCTC CTAGCGCAAA GTCTCCACAA TTGTGGATAC AAGATGAATG ATGTAGTGTC 240
GATCTGCGCC GAGAATAATA CAAGATTTT TATTCCTGTT ATTGCAGCTT GGTATATTGG 300
TATGATTGTA GCACCTGTTA ATGAAAGTTA CATCCCAGAT GAACTCTGTA AGGTCATGGG 360
TATATCGAAA CCACAAATAG TTTTTCGAC AAAGAACATT TTAAATAAGG TATTGGAGGT 420
ACAGAGCAGA ACTAATTTCA TAAAAAGGAT CATCGTACTT GATACTGTAG AAAACATACA 480
CGTTGTGAA AGTCTTCCCA ATTTTATTT TCCTTATTCG GATGGAAATA TTGCCAACTT 540
CAAACCTTTA CATTTTCGATC CTGTAGAGCA AGTGGCAGCT ATCTTATGTT CGTCAGGCAC 600
TACTGGATTA CCGAAAGGTG TAATGCAAAC TCACCAAAT ATTTGTGTCC GACTTATACA 660
TGCTTTAGAC CCCAGGGCAG GAACGCAACT TATTCCTGGT GTGACAGTCT TAGTATATCT 720
GCCTTTTTTC CATGCTTTTG GGTTCCTAT AACCTTGGGA TACTTCATGG TGGGTCTTCG 780
TGTTATCATG TCAAGACGAT TTGATCCAGA AGCATTCTTA AAAGCTATTC AGGATTATGA 840
AGTTCGAAGT GTAATTAACG TTCCATCAGT AATATTGTTT TTATCGAAAA GTCCTTTGGT 900
TGACAAATAC GATTTATCAA GTTTAAGGGA ATTGTGTTGC GGTGCGGCAC CATTAGCAA 960
AGAAAGTTGCT GAGGTTGCAG CAAAACGATT AAACCTGCCA GGAATTGCT GTGGATTGG 1020
TTTGACAGAA TCTACTTCAG CTAATATACA CAGTCTTAGG GATGAATTTA AACCAGGATC 1080
ACTTGAAGA GTTACTCCTT TAATGGCAGC TAAATAGCA GATAGGGAAA CTGGTAAAGC 1140
ATTGGGACCA AATCAAGTTG GTGAATTATG CATTAAGGT CCCATGGTAT CGAAAGGTTA 1200
CGTGAACAAT GTAGAAGCTA CCAAAGAAGC TATTGATGAT GATGGTTGGC TTCACTCTGG 1260
AGACTTTGGA TACTATGATG AGGATGAGCA TTTCTATGTG GTGGACCGTT ACAAGGAATT 1320
GATTAAATAT AAGGGCTCTC AGGTAGCACC TGCAGAACTA GAAGAGATTT TATTGAAAAA 1380
TCCATGTATC AGAGATGTTG CTGTGGTTGG TATTCCTGAT CTAGAAGCTG GAGAACTGCC 1440
ATCTGCGTTT GTGGTTAAAC AGCCCGGAAA GGAGATTACA GCTAAAGAAG TGTACGATTA 1500
TCTTGCCGAG AGGGTCTCCC ATACAAAGTA TTTGCGTGGA GGGGTTGAT TCGTTGATAG 1560
CATACCACGG AATGTTACAG GTAAAATTAC AAGAAAGGAA CTTCTGAAGC AGTTGCTGGA 1620
GAAGGCGGGA GGT

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FIG. 65

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LucPpl 81-0B11

E	D	L	T	A	G	E	M	L	F	R	A	L	R	K	H	S	H	L	P	L	18
Q	A	L	V	D	V	V	G	D	E	S	L	S	Y	K	E	F	F	E	A	38	
T	V	L	L	A	Q	S	L	H	N	C	G	Y	K	M	N	D	V	V	S	78	
I	C	A	E	N	N	T	R	F	F	I	P	V	I	A	A	W	Y	I	G	98	
M	I	V	A	P	V	N	E	S	Y	I	P	D	E	L	C	K	V	M	G	118	
I	S	K	P	Q	I	V	F	T	T	K	N	I	L	N	K	V	L	E	V	138	
Q	S	R	T	N	F	I	K	R	I	I	V	L	D	T	V	E	N	I	H	158	
G	C	E	S	L	P	N	F	I	S	R	Y	S	D	G	N	I	A	N	F	178	
K	P	L	H	E	D	P	V	E	Q	V	A	A	I	L	C	S	S	G	T	198	
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A	L	D	P	R	A	G	T	Q	L	I	P	G	V	T	V	L	V	Y	L	238	
P	F	F	H	A	F	G	F	S	I	T	L	G	Y	F	M	V	G	L	R	258	
V	I	M	S	R	R	F	D	P	E	A	F	L	K	A	I	Q	D	Y	E	278	
V	R	S	V	I	N	V	P	S	V	I	L	F	L	S	K	S	P	L	V	298	
D	K	Y	D	L	S	S	L	R	E	L	C	C	G	A	A	P	L	A	K	318	
E	V	A	E	V	A	A	K	R	L	N	L	P	G	I	R	C	G	F	G	338	
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L	G	P	N	Q	V	G	E	L	C	I	K	G	P	M	V	S	K	G	Y	398	
V	N	N	V	E	A	T	K	E	A	I	D	D	D	G	W	L	H	S	G	418	
D	F	G	Y	Y	D	E	D	E	H	F	Y	V	V	D	R	Y	K	E	L	438	
I	K	Y	K	G	S	Q	V	A	P	A	E	L	E	E	I	L	L	K	N	458	
P	C	I	R	D	V	K	A	V	V	G	I	P	D	L	E	A	G	E	L	478	
S	A	F	V	V	K	Q	P	G	K	Y	L	R	G	G	V	R	F	V	D	498	
I	P	R	N	V	T	G	K	I	T	R	K	E	L	L	K	Q	L	L	E	518	
K	A	G	G																	538	
																				542	

FIG. 66

1  
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<120> Thermostable luciferases and methods of  
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<130> 341.012WO1

<150> US 09/396,154

<151> 1999-09-15

<150> US 09/156,946

<151> 1998-09-18

<150> PCT/US98/19494

<151> 1998-09-18

<150> US 60/059,379

<151> 1997-09-19

<160> 93

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 1639

<212> DNA

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

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catagcattg acaaatgctc atacaaaaga aaatgtthtta tatgaagagt thttaaaatt	180
gtcgtgtcgt ttagcggaaa gthttaaaaa gtatggatta aaacaaaacg acacaatagc	240
ggtgtgtagc gaaaatggtt tgcaatthtt ccttcctata attgcatcat tgtatcttgg	300
aataattgca gcacctgtta gtgataaata cattgaacgt gaattaatac acagtcttgg	360
tattgtaaaa ccacgcataa thttttgctc caagaatact thtcaaaaag tactgaatgt	420
aaaatctaaa ttaaaatatg tagaaactat tattatatta gacttaaatg aagacttagg	480



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aggttatcaa tgcctcaaca actttatttc tcaaaattcc gatattaatc tggacgtaaa      540
aaaattttaa ccatattctt ttaatcgaga cgatcagggtt gcgttggtaa tgttttcttc      600
tggtacaact ggtgtttcga agggagtcac gctaactcac aagaatattg ttgcacgatt      660
ttctcttgca aaagatccta ctttttggtaa cgcaattaat ccaacgacag caattttaac      720
ggtaatacct ttccaccatg gtttttggtat gatgaccaca ttaggatact ttacttgtagg      780
attccgagtt gttctaatac acacgtttga agaaaaacta tttctacaat cattacaaga      840
ttataaagtg gaaagtactt tacttgtagc aacattaatg gcattttcttg caaaaagtgc      900
attagttgaa aagtacgatt tatcgactt aaaagaaatt gcacttggtg gcgcaccttt      960
atcaaaagaa attggggaga tggtgaaaaa acggttttaa ttaaactttg tcaggcaagg     1020
gtatggatta acagaaacca cttcggtgtg ttttaattaca ccgaacaatg acgtcagacc     1080
gggatcaact ggtaaaatag taccatttca cgctgtttaa gttgtcgatc ctacaacagg     1140
aaaaattttg gggccaaatg aacctggaga attgtatttt aaaggcgaca tgataatgaa     1200
aggttattat aataatgaag aagctactaa agcaattatt aacaaagacg gatgggtgag     1260
ctctgggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa     1320
gtcattaatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt     1380
acaacatccg tatattgttg atgccggcgt tactgggtata ccggatgaag ccgcgggcga     1440
gcttccagct gcagggtgtg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca     1500
aaattttgtt tccagtcaag tttcaacagc caaatggcta cgtgggtgggg tgaaattttt     1560
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tgaaaaacac accaatggg                                     1639

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<210> 2

<211> 1639

<212> DNA

<213> Artificial Sequence

<220>

<223> note = "mutant luciferase"

<400> 2

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catagcattg acaaatgctc atacaaaagc ccctgtttta tatgaagagt tgttaaaatt     180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc     240
ggtgtgtagc gaaaatgggt tgcaattttt ctttctata attgcatcat tgtatcttgg     300
aataattgca gcacctgtta gtgataaata cattgaacgt gaattaatac acagtcttgg     360
tattgtaaaa ccacgcataa ttttttgctc caagaatact tttcaaaaag tactgaatgt     420
aaaatctaaa ttaaaatatg tagaaactat tattatatta gacttaaatg aagacttagg     480
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aaaattttaa ccatattctt ttaatcgaga cgatcagggtt gcgttggtaa tgttttcttc     600
tggtacaact ggtgtttcga agggagtcac gctaactcac aagaatattg ttgcacgatt     660
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```

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ggtaataacct ttccaccatg gttttggtat gatgaccaca ttaggatact ttacttgtgg      780
attccgagtt gttctaatagc acacgtttga agaaaaacta tttctacaat cattacaaga      840
ttataaagtg gaaagtactt tacttgtacc aacattaatg gcattttttg caaaaagtgc      900
attagttgaa aagtacgatt tatcgcaactt aaaagaaatt gcacctggtg gcgcaccttt      960
atcaaaagaa attggggaga tggtgaaaaa acggttttaa ttaaactttg tcaggcaagg     1020
gtatggatta acagaaacca cttcggctgt ttttaattaca ccgaacaatg acgtcagacc     1080
gggatcaact ggtaaaatag taccatttca cgctgtttaa gttgtcgatc ctacaacagg     1140
aaaaattttg gggccaaatg aaactggaga attgtatttt aaaggcgaca tgataatgaa     1200
aggttattat aataatgaag aagctactaa agcaattatt aacaaagacg gatggttgcg     1260
ctctggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa     1320
gtcattaatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt     1380
acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga     1440
gcttccagct gcagggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca     1500
aaattttgtt tccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt     1560
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tgaaaaacac accaatggg                                1639

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&lt;210&gt; 3

&lt;211&gt; 1639

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; note = "mutant luciferase"

&lt;400&gt; 3

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catagcattg acaaatgctc atacaaaagc ccctgtttta tatgaagagt ttttaaaatt     180
gtcgtgtcgt ttagcggaaa gttttaaaaa gtatggatta aaacaaaacg acacaatagc     240
ggtgtgtagc gaaaatgggt tgcaattttt ccttcctata attgcatcat tgtatcttgg     300
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ctctgggtgat	attgcttatt	atgacaatga	tggccatttt	tatattgttg	acaggctgaa	1320
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acaacatccg	tatattgttg	atgccggcgt	tactgggtata	ccggatgaag	ccgcggggcga	1440
gcttccagct	gcagggtgtg	tagtacagac	tggaaaatat	ctaaacgaac	aaatcgtaca	1500
aaattttgtt	tccagtcaag	tttcaacagc	caaattggcta	cgtgggtggg	tgaaattttt	1560
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&lt;210&gt; 4

&lt;211&gt; 1639

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;400&gt; 4

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catagcattg	acaaatgctc	atacaaaagc	ccctgtttta	tatgaagagt	ttttaaaatt	180
gtcgtgtcgt	ttagcggaaa	gttttaaaaa	gtatggatta	aaacaaaacg	acacaatagc	240
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ttataaagtg	gaaagtactt	tacttgtacc	aacattaatg	gcattttttg	caaaaagtgc	900
attagttgaa	aagtacgatt	tatcgcaact	aaaagaaatt	gcactctggg	gcgcaccttt	960
atcaaaagaa	attggggaga	tggtgaaaaa	acggttttaa	ttaaactttg	tcaggcaagg	1020
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&lt;210&gt; 5

&lt;211&gt; 1639

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;400&gt; 5

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&lt;211&gt; 1639

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&lt;220&gt;

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&lt;210&gt; 11

&lt;211&gt; 1639

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;400&gt; 11

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&lt;210&gt; 12

&lt;211&gt; 1642

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;400&gt; 12

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catagcattg acaaattgctc atacaaaaga aaatgtttta tatgaagagt ttttaaaatt	180
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ggtgtgtagc gaaaatgggt tgcaatTTTT ccttccttta attgcatcat tgtatcctgg	300
aataattgca gcacctgtta gtgataaata cattgaacgt gaattaatac acagtcttgg	360
tattgtaaaa ccacgcataa ttttttgttc caagaatact tttcaaaaag tactgaatgt	420
aaaatctaaa ttaaaatatg tagaaactat tattatatta gacttaaatg aagacttagg	480
aggttatcaa tgctcaaca actttatttc tcaaaattcc gatattaatc ttgacgtaaa	540
aaaattttaa ccaaatctct ttaatcgaga cgatcagggt gcgttggtta tgttttcttc	600
tgggtacaact ggtgtttcga agggagtcac gctaactcac aagaatattg ttgcacgatt	660
ttctcattgc aaagatccta ctttttggtta cgcaattaat ccaacgacag caattttaac	720
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attagttgaa aagtacgatt tatcgcaact aaaagaaatt gcacctgggt gcgcaccttt	960
atcaaaagaa attggggaga tgggtgaaaa acggttttaa ttaaactttg tcaggcaagg	1020
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ctctgggtgat attgcttatt atgacaatga tggccatttt tatattgtgg acaggctgaa	1320
gtcattaatt aaatataaag gttatcaggt tgcacctgct gaaattgagg gaatactctt	1380
acaacatccg tatattgttg atgcggcgt tactgggtata ccggatgaag ccgcggggcga	1440
gcttcacgct gcagggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca	1500
aaattttgtt tccagtcaag tttcaacagc caaatggcta cgtgggtggg tgaaattttt	1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatgtt	1620
tgaaaaacac aaatctaagc tg	1642

&lt;210&gt; 13

&lt;211&gt; 1626

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;400&gt; 13

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ttagtagatg tgggtggcga cgaatcgctt tcctataaaag agttttttga agcgacagtc      180
ctcctagcgc aaagtctcca caattgtgga tacaagatga atgatgtagt gtcgatctgc      240
gccgagaata atacaagatt ttttattccc gttattgcag cttggtatat tgggtatgatt      300
gtagcacctg ttaatgaaag ttacatccca gatgaactct gtaaggatgat gggatatatcg      360
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agaactaatt tcataaaaag gatcatcata cttgatactg tagaaaacat acacggttgt      480
gaaagtcttc ccaattttat ttctcgttat tcggatggaa atattgccaa cttcaaacct      540
ttacatttcg atcctgttga gcaagtggca gctatcttat gttcgtcagg cactactgga      600
ttaccgaaag gtgtaatgca aactcaccaa aatatttgtg tccgacttat acatgcttta      660
gaccccgagg caggaacgca acttattcct ggtgtgacag tcttagtata tctgcctttt      720
ttccatgctt ttgggttctc tataaccttg ggatacttca tgggtgggtct tcgtgttatc      780
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agtgtaatga acgttccatc agtaatatg ttcttatcga aaagtccttt ggttgacaaa      900
tacgatttat caagttaag ggaatttgtg tgcggtgcgg caccattagc aaaagaagtt      960
gctgaggttg cagcaaaacg attaaacttg ccaggaattc gctgtggatt tggtttgaca     1020
gaatctactt cagctaatat acacagtctt agggatgaat ttaaatacagg atcacttgga     1080
agagttactc ctttaatggc agctaaaata gcagataggg aaactggtaa agcattggga     1140
ccaaatcaag ttggtgaatt atgcattaaa ggtcccatgg tatcgaaagg ttacgtgaac     1200
aatgtagaag ctaccaaaga agctattgat gatgatgggt ggcttcactc tggagacttt     1260
ggatactatg atgaggatga gcatttctat gtggtggacc gttacaagga attgattaaa     1320
tataagggct ctcaggtagc acctgcagaa ctagaagaga ttttattgaa aaatccatgt     1380
atcagagatg ttgctgtggt tggatttcct gatctagaag ctggagaact gccatctgcg     1440
tttgtgggta aacagcccgg aaaggagatt acagctaaag aagtgtacga ttatcttgcc     1500
gagaggggtc cccatacaaa gtatttgctg ggaggggttc gattcggtga tagcatacca     1560
aggaatgtta caggtaaaat tacaagaaag gaacttctga agcagttgct ggagaaggcg     1620
ggaggt                                           1626

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&lt;210&gt; 14

&lt;211&gt; 544

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;400&gt; 14

Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu

1

5

10

15

Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr

20

25

30

Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu  
 35 40 45  
 Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu  
 50 55 60  
 Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys  
 65 70 75 80  
 Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Ile Ile Ala Ser Leu Tyr  
 85 90 95  
 Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu  
 100 105 110  
 Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser  
 115 120 125  
 Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr  
 130 135 140  
 Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr  
 145 150 155 160  
 Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp  
 165 170 175  
 Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala  
 180 185 190  
 Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met  
 195 200 205  
 Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro  
 210 215 220  
 Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile  
 225 230 235 240  
 Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr  
 245 250 255  
 Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe  
 260 265 270  
 Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro  
 275 280 285  
 Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp  
 290 295 300  
 Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys  
 305 310 315 320  
 Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg  
 325 330 335  
 Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro  
 340 345 350  
 Asn Asn Asp Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His  
 355 360 365

Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn  
 370 375 380  
 Glu Pro Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Gly Tyr  
 385 390 395 400  
 Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asn Lys Asp Gly Trp  
 405 410 415  
 Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr  
 420 425 430  
 Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val  
 435 440 445  
 Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val  
 450 455 460  
 Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro  
 465 470 475 480  
 Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile  
 485 490 495  
 Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg  
 500 505 510  
 Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys  
 515 520 525  
 Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly  
 530 535 540

&lt;210&gt; 15

&lt;211&gt; 544

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;400&gt; 15

Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu  
 1 5 10 15  
 Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr  
 20 25 30  
 Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu  
 35 40 45  
 Asn Val Leu Tyr Glu Glu Leu Leu Lys Leu Ser Cys Arg Leu Ala Glu  
 50 55 60  
 Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys  
 65 70 75 80

Ser	Glu	Asn	Gly	Leu	Gln	Phe	Phe	Leu	Pro	Ile	Ile	Ala	Ser	Leu	Tyr			
				85					90					95				
Leu	Gly	Ile	Ile	Ala	Ala	Pro	Val	Ser	Asp	Lys	Tyr	Ile	Glu	Arg	Glu			
				100					105					110				
Leu	Ile	His	Ser	Leu	Gly	Ile	Val	Lys	Pro	Arg	Ile	Ile	Phe	Cys	Ser			
				115					120					125				
Lys	Asn	Thr	Phe	Gln	Lys	Val	Leu	Asn	Val	Lys	Ser	Lys	Leu	Lys	Tyr			
				130					135					140				
Val	Glu	Thr	Ile	Ile	Ile	Leu	Asp	Leu	Asn	Glu	Asp	Leu	Gly	Gly	Tyr			
				145					150					155				160
Gln	Cys	Leu	Asn	Asn	Phe	Ile	Ser	Gln	Asn	Ser	Asp	Ile	Asn	Leu	Asp			
				165					170					175				
Val	Lys	Lys	Phe	Lys	Pro	Tyr	Ser	Phe	Asn	Arg	Asp	Asp	Gln	Val	Ala			
				180					185					190				
Leu	Val	Met	Phe	Ser	Ser	Gly	Thr	Thr	Gly	Val	Ser	Lys	Gly	Val	Met			
				195					200					205				
Leu	Thr	His	Lys	Asn	Ile	Val	Ala	Arg	Phe	Ser	His	Ala	Lys	Asp	Pro			
				210					215					220				
Thr	Phe	Gly	Asn	Ala	Ile	Asn	Pro	Thr	Thr	Ala	Ile	Leu	Thr	Val	Ile			
				225					230					235				240
Pro	Phe	His	His	Gly	Phe	Gly	Met	Met	Thr	Thr	Leu	Gly	Tyr	Phe	Thr			
				245					250					255				
Cys	Gly	Phe	Arg	Val	Val	Leu	Met	His	Thr	Phe	Glu	Glu	Lys	Leu	Phe			
				260					265					270				
Leu	Gln	Ser	Leu	Gln	Asp	Tyr	Lys	Val	Glu	Ser	Thr	Leu	Leu	Val	Pro			
				275					280					285				
Thr	Leu	Met	Ala	Phe	Phe	Ala	Lys	Ser	Ala	Leu	Val	Glu	Lys	Tyr	Asp			
				290					295					300				
Leu	Ser	His	Leu	Lys	Glu	Ile	Ala	Ser	Gly	Gly	Ala	Pro	Leu	Ser	Lys			
				305					310					315				320
Glu	Ile	Gly	Glu	Met	Val	Lys	Lys	Arg	Phe	Lys	Leu	Asn	Phe	Val	Arg			
				325					330					335				
Gln	Gly	Tyr	Gly	Leu	Thr	Glu	Thr	Thr	Ser	Ala	Val	Leu	Ile	Thr	Pro			
				340					345					350				
Asn	Asn	Asp	Val	Arg	Pro	Gly	Ser	Thr	Gly	Lys	Ile	Val	Pro	Phe	His			
				355					360					365				
Ala	Val	Lys	Val	Val	Asp	Pro	Thr	Thr	Gly	Lys	Ile	Leu	Gly	Pro	Asn			
				370					375					380				
Glu	Thr	Gly	Glu	Leu	Tyr	Phe	Lys	Gly	Asp	Met	Ile	Met	Lys	Gly	Tyr			
				385					390					395				400
Tyr	Asn	Asn	Glu	Glu	Ala	Thr	Lys	Ala	Ile	Ile	Asn	Lys	Asp	Gly	Trp			
				405					410					415				

Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr  
                   420                                  425                                  430  
 Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val  
                   435                                  440                                  445  
 Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val  
                   450                                  455                                  460  
 Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro  
 465                                  470                                  475                                  480  
 Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile  
                                   485                                  490                                  495  
 Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg  
                                   500                                  505                                  510  
 Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys  
                   515                                  520                                  525  
 Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly  
                   530                                  535                                  540

<210> 16

<211> 544

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<400> 16

Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu  
 1                                  5                                  10                                  15  
 Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr  
                   20                                  25                                  30  
 Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu  
                   35                                  40                                  45  
 Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu  
                   50                                  55                                  60  
 Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys  
 65                                  70                                  75                                  80  
 Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Ile Ile Ala Ser Leu Tyr  
                   85                                  90                                  95  
 Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu  
                   100                                  105                                  110  
 Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser  
                   115                                  120                                  125



Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr  
 130 135 140  
 Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr  
 145 150 155 160  
 Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp  
 165 170 175  
 Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala  
 180 185 190  
 Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met  
 195 200 205  
 Leu Thr His Lys Asn Ile Val Val Arg Phe Ser Leu Ala Lys Asp Pro  
 210 215 220  
 Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile  
 225 230 235 240  
 Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr  
 245 250 255  
 Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe  
 260 265 270  
 Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro  
 275 280 285  
 Thr Leu Met Ala Phe Phe Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp  
 290 295 300  
 Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys  
 305 310 315 320  
 Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg  
 325 330 335  
 Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro  
 340 345 350  
 Asn Asn Asp Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His  
 355 360 365  
 Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn  
 370 375 380  
 Glu Thr Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Gly Tyr  
 385 390 395 400  
 Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Thr Lys Asp Gly Trp  
 405 410 415  
 Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr  
 420 425 430  
 Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val  
 435 440 445  
 Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val  
 450 455 460

Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro  
 465 470 475 480  
 Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile  
 485 490 495  
 Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg  
 500 505 510  
 Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys  
 515 520 525  
 Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly  
 530 535 540

<210> 17

<211> 544

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<400> 17

Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu  
 1 5 10 15  
 Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr  
 20 25 30  
 Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu  
 35 40 45  
 Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu  
 50 55 60  
 Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys  
 65 70 75 80  
 Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Ile Ile Ala Ser Leu Tyr  
 85 90 95  
 Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu  
 100 105 110  
 Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser  
 115 120 125  
 Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr  
 130 135 140  
 Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr  
 145 150 155 160  
 Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp  
 165 170 175



Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys  
515 520 525

Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly  
530 535 540

<210> 18

<211> 544

<212> PRT

<213> Artificial Sequence

$\langle 220 \rangle$

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<223> /note = "mutant luciferase"
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<400> 18

Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu  
1 5 10 15

Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr  
20 25 30

Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu  
35 40 45

Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu  
50 55 60

Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys  
65 70 75 80

Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Ile Ile Ala Ser Leu Tyr  
85 90 95

Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu  
100 105 110

Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser  
115 120 125

Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr  
130 135 140

Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr  
145 150 155 160

Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp  
165 170 175

Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala  
180 185 190

Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met  
195 200 205

Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser His Ala Lys Asp Pro  
 310 315 320

Thr	Phe	Gly	Asn	Ala	Ile	Asn	Pro	Thr	Thr	Ala	Ile	Leu	Thr	Val	Ile	225	230	235	240
Pro	Phe	His	His	Gly	Phe	Gly	Met	Met	Thr	Thr	Leu	Gly	Tyr	Phe	Thr	245	250	255	
Cys	Gly	Phe	Arg	Val	Val	Leu	Met	His	Thr	Phe	Glu	Glu	Lys	Leu	Phe	260	265	270	
Leu	Gln	Ser	Leu	Gln	Asp	Tyr	Lys	Val	Glu	Ser	Thr	Leu	Leu	Val	Pro	275	280	285	
Thr	Leu	Met	Ala	Phe	Phe	Ala	Lys	Ser	Ala	Leu	Val	Glu	Lys	Tyr	Asp	290	295	300	
Leu	Ser	His	Leu	Lys	Glu	Ile	Ala	Ser	Gly	Gly	Ala	Pro	Leu	Ser	Lys	305	310	315	320
Glu	Ile	Gly	Glu	Met	Val	Lys	Lys	Arg	Phe	Lys	Leu	Asn	Phe	Val	Arg	325	330	335	
Gln	Gly	Tyr	Gly	Leu	Thr	Glu	Thr	Thr	Ser	Ala	Val	Leu	Ile	Thr	Pro	340	345	350	
Asn	Asn	Asp	Val	Arg	Pro	Gly	Ser	Thr	Gly	Lys	Ile	Val	Pro	Phe	His	355	360	365	
Ala	Val	Lys	Val	Val	Asp	Pro	Thr	Thr	Gly	Lys	Ile	Leu	Gly	Pro	Asn	370	375	380	
Glu	Thr	Gly	Glu	Leu	Tyr	Phe	Lys	Gly	Asp	Met	Ile	Met	Lys	Gly	Tyr	385	390	395	400
Tyr	Asn	Asn	Glu	Glu	Ala	Thr	Lys	Ala	Ile	Ile	Asn	Lys	Asp	Gly	Trp	405	410	415	
Leu	Arg	Ser	Gly	Asp	Ile	Ala	Tyr	Tyr	Asp	Asn	Asp	Gly	His	Phe	Tyr	420	425	430	
Ile	Val	Asp	Arg	Leu	Lys	Ser	Leu	Ile	Lys	Tyr	Lys	Gly	Tyr	Gln	Val	435	440	445	
Ala	Pro	Ala	Glu	Ile	Glu	Gly	Ile	Leu	Leu	Gln	His	Pro	Tyr	Ile	Val	450	455	460	
Asp	Ala	Gly	Val	Thr	Gly	Ile	Pro	Asp	Glu	Ala	Ala	Gly	Glu	Leu	Pro	465	470	475	480
Ala	Ala	Gly	Val	Val	Val	Gln	Thr	Gly	Lys	Tyr	Leu	Asn	Glu	Gln	Ile	485	490	495	
Val	Gln	Asn	Phe	Val	Ser	Ser	Gln	Val	Ser	Thr	Ala	Lys	Trp	Leu	Arg	500	505	510	
Gly	Gly	Val	Lys	Phe	Leu	Asp	Glu	Ile	Pro	Lys	Gly	Ser	Thr	Gly	Lys	515	520	525	
Ile	Asp	Arg	Lys	Val	Leu	Arg	Gln	Met	Phe	Glu	Lys	His	Thr	Asn	Gly	530	535	540	

&lt;211&gt; 544

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;400&gt; 19

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Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
 1             5             10             15
Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr
          20             25             30
Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
          35             40             45
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
          50             55             60
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65             70             75             80
Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr
          85             90             95
Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
          100            105            110
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
          115            120            125
Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Ser
          130            135            140
Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145            150            155            160
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ser Asn Leu Asp
          165            170            175
Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
          180            185            190
Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Pro Lys Gly Val Met
          195            200            205
Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro
          210            215            220
Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225            230            235            240
Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
          245            250            255
Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
          260            265            270

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Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
      275                      280                      285
Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
      290                      295                      300
Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
305                      310                      315                      320
Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg
      325                      330                      335
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro
      340                      345                      350
Lys Gly Asp Ala Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His
      355                      360                      365
Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn
      370                      375                      380
Glu Pro Gly Glu Leu Tyr Phe Lys Gly Ala Met Ile Met Lys Gly Tyr
385                      390                      395                      400
Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Asn Asp Gly Trp
      405                      410                      415
Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
      420                      425                      430
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
      435                      440                      445
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
      450                      455                      460
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465                      470                      475                      480
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
      485                      490                      495
Val Gln Asp Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
      500                      505                      510
Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
      515                      520                      525
Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
      530                      535                      540

```

&lt;210&gt; 20

&lt;211&gt; 544

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;221&gt; unsure

&lt;222&gt; (354)...(355)

&lt;223&gt; /note = "unknown amino acids"

&lt;400&gt; 20

```

Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
 1             5             10             15
Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr
             20             25             30
Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
             35             40             45
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
             50             55             60
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65             70             75             80
Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr
             85             90             95
Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
             100            105            110
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
             115            120            125
Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr
             130            135            140
Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145            150            155            160
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp
             165            170            175
Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
             180            185            190
Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Pro Lys Gly Val Met
             195            200            205
Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro
             210            215            220
Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225            230            235            240
Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
             245            250            255
Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
             260            265            270
Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
             275            280            285
Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp

```



290	295	300
Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys		
305	310	315
Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg		320
	325	330
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro		335
	340	345
Lys Xaa Xaa Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His		350
	355	360
Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn		365
	370	375
Glu Pro Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Gly Tyr		380
385	390	395
Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Lys Asp Gly Trp		400
	405	410
Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr		415
	420	425
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val		430
	435	440
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val		445
	450	455
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro		460
465	470	475
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile		480
	485	490
Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg		495
	500	505
Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys		510
	515	520
Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly		525
530	535	540

&lt;210&gt; 21

&lt;211&gt; 544

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;221&gt; unsure

&lt;222&gt; (354) ... (355)

<223> /note = "unknown amino acids"

<400> 21

```

Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
 1           5           10           15
Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr
          20           25           30
Ala Asp Ile Pro Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
          35           40           45
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
          50           55           60
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65           70           75           80
Ser Glu Asn Gly Leu Gln Tyr Phe Leu Pro Val Ile Ala Ser Leu Tyr
          85           90           95
Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu
          100          105          110
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
          115          120          125
Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr
          130          135          140
Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
          145          150          155          160
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp
          165          170          175
Val Lys Lys Phe Lys Pro Asn Ser Phe Asn Arg Asp Asp Gln Val Ala
          180          185          190
Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Pro Lys Gly Val Met
          195          200          205
Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Ile Ala Lys Asp Pro
          210          215          220
Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
          225          230          235          240
Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
          245          250          255
Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
          260          265          270
Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
          275          280          285
Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
          290          295          300
Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys

```

```

305          310          315          320
Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg
          325          330          335
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro
          340          345          350
Lys Xaa Xaa Ala Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His
          355          360          365
Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn
          370          375          380
Glu Pro Gly Glu Leu Tyr Phe Lys Gly Ala Met Ile Met Lys Gly Tyr
385          390          395          400
Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Lys Asp Gly Trp
          405          410          415
Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
          420          425          430
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
          435          440          445
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
          450          455          460
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465          470          475          480
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
          485          490          495
Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
          500          505          510
Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
          515          520          525
Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
530          535          540

```

<210> 22

<211> 544

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<221> unsure

<222> (354)...(355)

<223> /note = "unknown amino acids"

&lt;400&gt; 22

```

Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
 1             5             10             15
Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr
      20             25             30
Ala Asp Ile Pro Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
      35             40             45
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
      50             55             60
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65             70             75             80
Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr
      85             90             95
Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Val Glu Arg Glu
      100            105            110
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser
      115            120            125
Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr
      130            135            140
Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr
145            150            155            160
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ser Asn Leu Asp
      165            170            175
Val Lys Lys Phe Lys Pro Asn Ser Phe Asn Arg Asp Asp Gln Val Ala
      180            185            190
Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Pro Lys Gly Val Met
      195            200            205
Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro
      210            215            220
Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile
225            230            235            240
Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr
      245            250            255
Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe
      260            265            270
Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro
      275            280            285
Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp
      290            295            300
Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys
305            310            315            320
Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg

```

```

          325          330          335
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro
          340          345          350
Lys Xaa Xaa Ala Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His
          355          360          365
Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn
          370          375          380
Glu Thr Gly Glu Leu Tyr Phe Lys Gly Ala Met Ile Met Lys Gly Tyr
385          390          395          400
Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Lys Asp Gly Trp
          405          410          415
Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
          420          425          430
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
          435          440          445
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
          450          455          460
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465          470          475          480
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
          485          490          495
Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
          500          505          510
Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
          515          520          525
Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
          530          535          540

```

&lt;210&gt; 23

&lt;211&gt; 544

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;221&gt; unsure

&lt;222&gt; (354)...(355)

&lt;223&gt; /note = "unknown amino acids"

&lt;400&gt; 23

Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu

1	5	10	15
Ala Asp Gly Thr	Ala Gly Glu Gln Met	Phe Asp Ala Leu Ser Arg Tyr	
20	25	30	
Ala Asp Ile Pro Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu			
35	40	45	
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu			
50	55	60	
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys			
65	70	75	80
Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr			
85	90	95	
Leu Gly Ile Ile Val Ala Pro Val Asn Asp Lys Tyr Ile Glu Arg Glu			
100	105	110	
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser			
115	120	125	
Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Ser			
130	135	140	
Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr			
145	150	155	160
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp			
165	170	175	
Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala			
180	185	190	
Leu Ile Met Phe Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met			
195	200	205	
Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro			
210	215	220	
Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile			
225	230	235	240
Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr			
245	250	255	
Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe			
260	265	270	
Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro			
275	280	285	
Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp			
290	295	300	
Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys			
305	310	315	320
Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg			
325	330	335	
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro			

```

          340          345          350
Lys Xaa Xaa Ala Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His
          355          360          365
Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn
          370          375          380
Glu Pro Gly Glu Leu Tyr Phe Lys Gly Pro Met Ile Met Lys Gly Tyr
385          390          395          400
Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Asn Asp Gly Trp
          405          410          415
Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
          420          425          430
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
          435          440          445
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
          450          455          460
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465          470          475          480
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
          485          490          495
Val Gln Asp Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
          500          505          510
Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
          515          520          525
Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
          530          535          540

```

&lt;210&gt; 24

&lt;211&gt; 544

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;400&gt; 24

```

Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
 1          5          10          15
Glu Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr
          20          25          30
Ala Asp Ile Pro Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
          35          40          45
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu

```

50	55	60
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys		
65	70	75
Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr		80
	85	90
Leu Gly Ile Ile Val Ala Pro Val Asn Asp Lys Tyr Ile Glu Arg Glu		95
	100	105
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Val Phe Cys Ser		110
	115	120
Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Ser		125
	130	135
Ile Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr		140
145	150	155
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ser Asn Leu Asp		160
	165	170
Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala		175
	180	185
Leu Ile Met Phe Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met		190
	195	200
Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Leu Ala Lys Asp Pro		205
	210	215
Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile		220
225	230	235
Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr		240
	245	250
Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe		255
	260	265
Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro		270
	275	280
Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp		285
	290	295
Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys		300
305	310	315
Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg		320
	325	330
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro		335
	340	345
Lys Gly Asp Ala Lys Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His		350
	355	360
Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn		365
	370	375
Glu Pro Gly Glu Leu Tyr Phe Lys Gly Pro Met Ile Met Lys Gly Tyr		380



```

385              390              395              400
Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Asn Asp Gly Trp
              405              410              415
Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr
              420              425              430
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val
              435              440              445
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
              450              455              460
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465              470              475              480
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
              485              490              495
Val Gln Asp Tyr Val Ala Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
              500              505              510
Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
              515              520              525
Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Thr Asn Gly
              530              535              540

```

<210> 25

<211> 545

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<400> 25

```

Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
 1              5              10              15
Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr
              20              25              30
Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
              35              40              45
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
              50              55              60
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65              70              75              80
Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Leu Ile Ala Ser Leu Tyr
              85              90              95
Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu

```

100	105	110
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser		
115	120	125
Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr		
130	135	140
Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr		
145	150	155
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp		
165	170	175
Val Lys Lys Phe Lys Pro Asn Ser Phe Asn Arg Asp Asp Gln Val Ala		
180	185	190
Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met		
195	200	205
Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser His Cys Lys Asp Pro		
210	215	220
Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile		
225	230	235
Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr		
245	250	255
Cys Gly Phe Arg Val Ala Leu Met His Thr Phe Glu Glu Lys Leu Phe		
260	265	270
Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro		
275	280	285
Thr Leu Met Ala Phe Phe Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp		
290	295	300
Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys		
305	310	315
Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg		
325	330	335
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro		
340	345	350
Asp Thr Asp Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His		
355	360	365
Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn		
370	375	380
Glu Thr Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Gly Tyr		
385	390	395
Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asn Lys Asp Gly Trp		
405	410	415
Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr		
420	425	430
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val		

```

      435              440              445
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val
      450              455              460
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro
465              470              475              480
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile
              485              490              495
Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg
              500              505              510
Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys
              515              520              525
Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Lys Ser Lys
              530              535              540
Leu
545

```

&lt;210&gt; 26

&lt;211&gt; 542

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; note = "mutant luciferase"

&lt;400&gt; 26

```

Met Met Lys Arg Glu Lys Asn Val Ile Tyr Gly Pro Glu Pro Leu His
 1              5              10              15
Pro Leu Glu Asp Leu Thr Ala Gly Glu Met Leu Phe Arg Ala Leu Arg
              20              25              30
Lys His Ser His Leu Pro Gln Ala Leu Val Asp Val Val Gly Asp Glu
              35              40              45
Ser Leu Ser Tyr Lys Glu Phe Phe Glu Ala Thr Val Leu Leu Ala Gln
              50              55              60
Ser Leu His Asn Cys Gly Tyr Lys Met Asn Asp Val Val Ser Ile Cys
65              70              75              80
Ala Glu Asn Asn Thr Arg Phe Phe Ile Pro Val Ile Ala Ala Trp Tyr
              85              90              95
Ile Gly Met Ile Val Ala Pro Val Asn Glu Ser Tyr Ile Pro Asp Glu
              100              105              110
Leu Cys Lys Val Met Gly Ile Ser Lys Pro Gln Ile Val Phe Thr Thr
              115              120              125
Lys Asn Ile Leu Asn Lys Val Leu Glu Val Gln Ser Arg Thr Asn Phe

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130	135	140
Ile Lys Arg Ile Ile Ile	Leu Asp Thr Val Glu Asn Ile His Gly Cys	
145	150	155
Glu Ser Leu Pro Asn Gly Ile Ser Arg Tyr Ser Asp Gly Asn Ile Ala		160
	165	170
Asn Phe Lys Pro Leu His Phe Asp Pro Val Glu Gln Val Ala Ala Ile		175
	180	185
Leu Cys Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met Gln Thr		190
	195	200
His Gln Asn Ile Cys Val Arg Leu Ile His Ala Leu Asp Pro Arg Ala		205
	210	215
Gly Thr Gln Leu Ile Pro Gly Val Thr Val Leu Val Tyr Leu Pro Phe		220
225	230	235
Phe His Ala Phe Gly Phe Ser Ile Thr Leu Gly Tyr Phe Met Val Gly		240
	245	250
Leu Arg Val Ile Met Phe Arg Arg Phe Asp Gln Glu Ala Phe Leu Lys		255
	260	265
Ala Ile Gln Asp Tyr Glu Val Arg Ser Val Ile Asn Val Pro Ser Val		270
	275	280
Ile Leu Phe Leu Ser Lys Ser Pro Leu Val Asp Lys Tyr Asp Leu Ser		285
	290	295
Ser Leu Arg Glu Leu Cys Cys Gly Ala Ala Pro Leu Ala Lys Glu Val		300
305	310	315
Ala Glu Val Ala Ala Lys Arg Leu Asn Leu Pro Gly Ile Arg Cys Gly		320
	325	330
Phe Gly Leu Thr Glu Ser Thr Ser Ala Asn Ile His Ser Leu Arg Asp		335
	340	345
Glu Phe Lys Ser Gly Ser Leu Gly Arg Val Thr Pro Leu Met Ala Ala		350
	355	360
Lys Ile Ala Asp Arg Glu Thr Gly Lys Ala Leu Gly Pro Asn Gln Val		365
	370	375
Gly Glu Leu Cys Ile Lys Gly Pro Met Val Ser Lys Gly Tyr Val Asn		380
385	390	395
Asn Val Glu Ala Thr Lys Glu Ala Ile Asp Asp Asp Gly Trp Leu His		400
	405	410
Ser Gly Asp Phe Gly Tyr Tyr Asp Glu Asp Glu His Phe Tyr Val Val		415
	420	425
Asp Arg Tyr Lys Glu Leu Ile Lys Tyr Lys Gly Ser Gln Val Ala Pro		430
	435	440
Ala Glu Leu Glu Glu Ile Leu Leu Lys Asn Pro Cys Ile Arg Asp Val		445
	450	455
Ala Val Val Gly Ile Pro Asp Leu Glu Ala Gly Glu Leu Pro Ser Ala		460

465                                      470                                      475                                      480  
 Phe Val Val Lys Gln Pro Gly Lys Glu Ile Thr Ala Lys Glu Val Tyr  
    485                                      490                                      495  
 Asp Tyr Leu Ala Glu Arg Val Ser His Thr Lys Tyr Leu Arg Gly Gly  
    500                                      505                                      510  
 Val Arg Phe Val Asp Ser Ile Pro Arg Asn Val Thr Gly Lys Ile Thr  
    515                                      520                                      525  
 Arg Lys Glu Leu Leu Lys Gln Leu Leu Glu Lys Ala Gly Gly  
    530                                      535                                      540

<210> 27

<211> 548

<212> PRT

<213> *Luciola cruciata*

<400> 27

Met Glu Asn Met Glu Asn Asp Glu Asn Ile Val Val Gly Pro Lys Pro  
 1                                      5                                      10                                      15  
 Phe Tyr Pro Ile Glu Glu Gly Ser Ala Gly Thr Gln Leu Arg Lys Tyr  
    20                                      25                                      30  
 Met Glu Arg Tyr Ala Lys Leu Gly Ala Ile Ala Phe Thr Asn Ala Val  
    35                                      40                                      45  
 Thr Gly Val Asp Tyr Ser Tyr Ala Glu Tyr Leu Glu Lys Ser Cys Cys  
    50                                      55                                      60  
 Leu Gly Lys Ala Leu Gln Asn Tyr Gly Leu Val Val Asp Gly Arg Ile  
 65                                      70                                      75                                      80  
 Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe Phe Ile Pro Val Ile Ala  
    85                                      90                                      95  
 Gly Leu Phe Ile Gly Val Gly Val Ala Pro Thr Asn Glu Ile Tyr Thr  
    100                                      105                                      110  
 Leu Arg Glu Leu Val His Ser Leu Gly Ile Ser Lys Pro Thr Ile Val  
    115                                      120                                      125  
 Phe Ser Ser Lys Lys Gly Leu Asp Lys Val Ile Thr Val Gln Lys Thr  
    130                                      135                                      140  
 Val Thr Thr Ile Lys Thr Ile Val Ile Leu Asp Ser Lys Val Asp Tyr  
 145                                      150                                      155                                      160  
 Arg Gly Tyr Gln Cys Leu Asp Thr Phe Ile Lys Arg Asn Thr Pro Pro  
    165                                      170                                      175  
 Gly Phe Gln Ala Ser Ser Phe Lys Thr Val Glu Val Asp Arg Lys Glu  
    180                                      185                                      190  
 Gln Val Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys  
    195                                      200                                      205

Gly Val Gln Leu Thr His Glu Asn Thr Val Thr Arg Phe Ser His Ala			
210	215	220	
Arg Asp Pro Ile Tyr Gly Asn Gln Val Ser Pro Gly Thr Ala Val Leu			
225	230	235	240
Thr Val Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly			
	245	250	255
Tyr Leu Ile Cys Gly Phe Arg Val Val Met Leu Thr Lys Phe Asp Glu			
	260	265	270
Glu Thr Phe Leu Lys Thr Leu Gln Asp Tyr Lys Cys Thr Ser Val Ile			
	275	280	285
Leu Val Pro Thr Leu Phe Ala Ile Leu Asn Lys Ser Glu Leu Leu Asn			
	290	295	300
Lys Tyr Asp Leu Ser Asn Leu Val Glu Ile Ala Ser Gly Gly Ala Pro			
305	310	315	320
Leu Ser Lys Glu Val Gly Glu Ala Val Ala Arg Arg Phe Asn Leu Pro			
	325	330	335
Gly Val Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Ile			
	340	345	350
Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala Ser Gly Lys Val Val			
	355	360	365
Pro Leu Phe Lys Ala Lys Val Ile Asp Leu Asp Thr Lys Lys Ser Leu			
	370	375	380
Gly Pro Asn Arg Arg Gly Glu Val Cys Val Lys Gly Pro Met Leu Met			
385	390	395	400
Lys Gly Tyr Val Asn Asn Pro Glu Ala Thr Lys Glu Leu Ile Asp Glu			
	405	410	415
Glu Gly Trp Leu His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu Glu Lys			
	420	425	430
His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly			
	435	440	445
Tyr Gln Val Pro Pro Ala Glu Leu Glu Ser Val Leu Leu Gln His Pro			
	450	455	460
Ser Ile Phe Asp Ala Gly Val Ala Gly Val Pro Asp Pro Val Ala Gly			
465	470	475	480
Glu Leu Pro Gly Ala Val Val Val Leu Glu Ser Gly Lys Asn Met Thr			
	485	490	495
Glu Lys Glu Val Met Asp Tyr Val Ala Ser Gln Val Ser Asn Ala Lys			
	500	505	510
Arg Leu Arg Gly Gly Val Arg Phe Val Asp Glu Val Pro Lys Gly Leu			
	515	520	525
Thr Gly Lys Ile Asp Gly Arg Ala Ile Arg Glu Ile Leu Lys Lys Pro			
	530	535	540

Val Ala Lys Met

545

&lt;210&gt; 28

&lt;211&gt; 548

&lt;212&gt; PRT

<213> *Luciola lateralis*

&lt;400&gt; 28

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Met Glu Asn Met Glu Asn Asp Glu Asn Ile Val Tyr Gly Pro Glu Pro
 1             5             10             15
Phe Tyr Pro Ile Glu Glu Gly Ser Ala Gly Ala Gln Leu Arg Lys Tyr
      20             25             30
Met Asp Arg Tyr Ala Lys Leu Gly Ala Ile Ala Phe Thr Asn Ala Leu
      35             40             45
Thr Gly Val Asp Tyr Thr Tyr Ala Glu Tyr Leu Glu Lys Ser Cys Cys
 50             55             60
Leu Gly Glu Ala Leu Lys Asn Tyr Gly Leu Val Val Asp Gly Arg Ile
65             70             75             80
Ala Leu Cys Ser Glu Asn Cys Glu Glu Phe Phe Ile Pro Val Leu Ala
      85             90             95
Gly Leu Phe Ile Gly Val Gly Val Ala Pro Thr Asn Glu Ile Tyr Thr
      100            105            110
Leu Arg Glu Leu Val His Ser Leu Gly Ile Ser Lys Pro Thr Ile Val
      115            120            125
Phe Ser Ser Lys Lys Gly Leu Asp Lys Val Ile Thr Val Gln Lys Thr
      130            135            140
Val Ala Thr Ile Lys Thr Ile Val Ile Leu Asp Ser Lys Val Asp Tyr
      145            150            155            160
Arg Gly Tyr Gln Ser Met Asp Asn Phe Ile Lys Lys Asn Thr Pro Gln
      165            170            175
Gly Phe Lys Gly Ser Ser Phe Lys Thr Val Glu Val Asn Arg Lys Glu
      180            185            190
Gln Val Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys
      195            200            205
Gly Val Gln Leu Thr His Glu Asn Ala Val Thr Arg Phe Ser His Ala
      210            215            220
Arg Asp Pro Ile Tyr Gly Asn Gln Val Ser Pro Gly Thr Ala Ile Leu
      225            230            235            240
Thr Val Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly
      245            250            255
Tyr Leu Thr Cys Gly Phe Arg Ile Val Met Leu Thr Lys Phe Asp Glu

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                260                265                270
Glu Thr Phe Leu Lys Thr Leu Gln Asp Tyr Lys Cys Ser Ser Val Ile
      275                280                285
Leu Val Pro Thr Leu Phe Ala Ile Leu Asn Arg Ser Glu Leu Leu Asp
      290                295                300
Lys Tyr Asp Leu Ser Asn Leu Val Glu Ile Ala Ser Gly Gly Ala Pro
305                310                315                320
Leu Ser Lys Glu Ile Gly Glu Ala Val Ala Arg Arg Phe Asn Leu Pro
      325                330                335
Gly Val Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Ile
      340                345                350
Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala Ser Gly Lys Val Val
      355                360                365
Pro Leu Phe Lys Ala Lys Val Ile Asp Leu Asp Thr Lys Lys Thr Leu
      370                375                380
Gly Pro Asn Arg Arg Gly Glu Val Cys Val Lys Gly Pro Met Leu Met
385                390                395                400
Lys Gly Tyr Val Asp Asn Pro Glu Ala Thr Arg Glu Ile Ile Asp Glu
      405                410                415
Glu Gly Trp Leu His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu Glu Lys
      420                425                430
His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly
      435                440                445
Tyr Gln Val Pro Pro Ala Glu Leu Glu Ser Val Leu Leu Gln His Pro
      450                455                460
Asn Ile Phe Asp Ala Gly Val Ala Gly Val Pro Asp Pro Ile Ala Gly
465                470                475                480
Glu Leu Pro Gly Ala Val Val Val Leu Glu Lys Gly Lys Ser Met Thr
      485                490                495
Glu Lys Glu Val Met Asp Tyr Val Ala Ser Gln Val Ser Asn Ala Lys
      500                505                510
Arg Leu Arg Gly Gly Val Arg Phe Val Asp Glu Val Pro Lys Gly Leu
      515                520                525
Thr Gly Lys Ile Asp Gly Lys Ala Ile Arg Glu Ile Leu Lys Lys Pro
      530                535                540
Val Ala Lys Met
545

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&lt;210&gt; 29

&lt;211&gt; 548

&lt;212&gt; PRT

<213> *Luciola mingrelica*



&lt;400&gt; 29

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Met Glu Met Glu Lys Glu Glu Asn Val Val Tyr Gly Pro Leu Pro Phe
 1              5              10              15
Tyr Pro Ile Glu Glu Gly Ser Ala Gly Ile Gln Leu His Lys Tyr Met
      20              25              30
His Gln Tyr Ala Lys Leu Gly Ala Ile Ala Phe Ser Asn Ala Leu Thr
      35              40              45
Gly Val Asp Ile Ser Tyr Gln Glu Tyr Phe Asp Ile Thr Cys Arg Leu
      50              55              60
Ala Glu Ala Met Lys Asn Phe Gly Met Lys Pro Glu Glu His Ile Ala
65              70              75              80
Leu Cys Ser Glu Asn Cys Glu Glu Phe Phe Ile Pro Val Leu Ala Gly
      85              90              95
Leu Tyr Ile Gly Val Ala Val Ala Pro Thr Asn Glu Ile Tyr Thr Leu
      100             105             110
Arg Glu Leu Asn His Ser Leu Gly Ile Ala Gln Pro Thr Ile Val Phe
      115             120             125
Ser Ser Arg Lys Gly Leu Pro Lys Val Leu Glu Val Gln Lys Thr Val
      130             135             140
Thr Cys Ile Lys Lys Ile Val Ile Leu Asp Ser Lys Val Asn Phe Gly
145             150             155             160
Gly His Asp Cys Met Glu Thr Phe Ile Lys Lys His Val Glu Leu Gly
      165             170             175
Phe Gln Pro Ser Ser Phe Val Pro Ile Asp Val Lys Asn Arg Lys Gln
      180             185             190
His Val Ala Leu Leu Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys
      195             200             205
Gly Val Arg Ile Thr His Glu Gly Ala Val Thr Arg Phe Ser His Ala
      210             215             220
Lys Asp Pro Ile Tyr Gly Asn Gln Val Ser Pro Gly Thr Ala Ile Leu
225             230             235             240
Thr Val Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly
      245             250             255
Tyr Phe Ala Cys Gly Tyr Arg Val Val Met Leu Thr Lys Phe Asp Glu
      260             265             270
Glu Leu Phe Leu Arg Thr Leu Gln Asp Tyr Lys Cys Thr Ser Val Ile
      275             280             285
Leu Val Pro Thr Leu Phe Ala Ile Leu Asn Lys Ser Glu Leu Ile Asp
      290             295             300
Lys Phe Asp Leu Ser Asn Leu Thr Glu Ile Ala Ser Gly Gly Ala Pro
305             310             315             320
Leu Ala Lys Glu Val Gly Glu Ala Val Ala Arg Arg Phe Asn Leu Pro

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325 330 335  
 Gly Val Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Phe Ile  
 340 345 350  
 Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala Ser Gly Lys Val Val  
 355 360 365  
 Pro Leu Phe Lys Val Lys Val Ile Asp Leu Asp Thr Lys Lys Thr Leu  
 370 375 380  
 Gly Val Asn Arg Arg Gly Glu Ile Cys Val Lys Gly Pro Ser Leu Met  
 385 390 395 400  
 Leu Gly Tyr Ser Asn Asn Pro Glu Ala Thr Arg Glu Thr Ile Asp Glu  
 405 410 415  
 Glu Gly Trp Leu His Thr Gly Asp Ile Gly Tyr Tyr Asp Glu Asp Glu  
 420 425 430  
 His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly  
 435 440 445  
 Tyr Gln Val Pro Pro Ala Glu Leu Glu Ser Val Leu Leu Gln His Pro  
 450 455 460  
 Asn Ile Phe Asp Ala Gly Val Ala Gly Val Pro Asp Pro Asp Ala Gly  
 465 470 475 480  
 Glu Leu Pro Gly Ala Val Val Val Met Glu Lys Gly Lys Thr Met Thr  
 485 490 495  
 Glu Lys Glu Ile Val Asp Tyr Val Asn Ser Gln Val Val Asn His Lys  
 500 505 510  
 Arg Leu Arg Gly Gly Val Arg Phe Val Asp Glu Val Pro Lys Gly Leu  
 515 520 525  
 Thr Gly Lys Ile Asp Ala Lys Val Ile Arg Glu Ile Leu Lys Lys Pro  
 530 535 540  
 Gln Ala Lys Met  
 545

&lt;210&gt; 30

&lt;211&gt; 548

&lt;212&gt; PRT

&lt;213&gt; Pyrocoelia miyako

&lt;400&gt; 30

Met Glu Asp Asp Ser Lys His Ile Met His Gly His Arg His Ser Ile  
 1 5 10 15  
 Leu Trp Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys  
 20 25 30  
 Arg Tyr Ala Gln Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ala  
 35 40 45



Val Asn Gln Arg Gly Glu Leu Cys Val Lys Gly Pro Met Ile Met Lys  
 385 390 395 400  
 Gly Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp  
 405 410 415  
 Gly Trp Leu His Ser Gly Asp Ile Ala Tyr Tyr Asp Lys Asp Gly His  
 420 425 430  
 Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr  
 435 440 445  
 Gln Val Pro Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Phe  
 450 455 460  
 Ile Phe Asp Ala Gly Val Ala Gly Ile Pro Asp Pro Asp Ala Gly Glu  
 465 470 475 480  
 Leu Pro Ala Ala Val Val Val Leu Glu Glu Gly Lys Met Met Thr Glu  
 485 490 495  
 Gln Glu Val Met Asp Tyr Val Ala Gly Gln Val Thr Ala Ser Lys Arg  
 500 505 510  
 Leu Arg Gly Gly Val Lys Phe Val Asp Glu Val Pro Lys Gly Leu Thr  
 515 520 525  
 Gly Lys Ile Asp Ser Arg Lys Ile Arg Glu Ile Leu Thr Met Gly Gln  
 530 535 540  
 Lys Ser Lys Leu  
 545

<210> 31  
 <211> 550  
 <212> PRT  
 <213> *Photinus pyralis*

<400> 31  
 Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr Pro  
 1 5 10 15  
 Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg  
 20 25 30  
 Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu  
 35 40 45  
 Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala  
 50 55 60  
 Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val  
 65 70 75 80  
 Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu  
 85 90 95  
 Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg

100	105	110
Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val		
115	120	125
Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro		
130	135	140
Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly		
145	150	155
Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe		
165	170	175
Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp Lys Thr Ile		
180	185	190
Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val		
195	200	205
Ala Leu Pro His Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp		
210	215	220
Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val		
225	230	235
Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu		
245	250	255
Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Glu Glu Leu		
260	265	270
Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val		
275	280	285
Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr		
290	295	300
Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser		
305	310	315
Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile		
325	330	335
Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr		
340	345	350
Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe		
355	360	365
Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val		
370	375	380
Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly		
385	390	395
Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly		
405	410	415
Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe		
420	425	430
Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln		

435	440	445
Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile		
450	455	460
Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu		
465	470	475
Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys		
	485	490
Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu		
	500	505
Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly		
	515	520
Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys		
	530	540
Gly Gly Lys Ser Lys Leu		
545	550	

&lt;210&gt; 32

&lt;211&gt; 547

&lt;212&gt; PRT

&lt;213&gt; Lampyris noctiluca

&lt;400&gt; 32

Met Glu Asp Ala Lys Asn Ile Met His Gly Pro Ala Pro Phe Tyr Pro		
1	5	10
Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg		
	20	30
Tyr Ala Gln Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ala Glu		
	35	45
Val Asn Ile Thr Tyr Ser Glu Tyr Phe Glu Met Ala Cys Arg Leu Ala		
	50	60
Glu Thr Met Lys Arg Tyr Gly Leu Gly Leu Gln His His Ile Ala Val		
	65	75
Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Cys Gly Ala Leu		
	85	95
Phe Ile Gly Val Gly Val Ala Ser Thr Asn Asp Ile Tyr Asn Glu Arg		
	100	110
Glu Leu Tyr Asn Ser Leu Ser Ile Ser Gln Pro Thr Ile Val Ser Cys		
	115	125
Ser Lys Arg Ala Leu Gln Lys Ile Leu Gly Val Gln Lys Lys Leu Pro		
	130	140
Ile Ile Gln Lys Ile Val Ile Leu Asp Ser Arg Glu Asp Tyr Met Gly		
	145	155
		160

Lys Gln Ser Met Tyr Ser Phe Ile Glu Ser His Leu Pro Ala Gly Phe  
 165 170 175  
 Asn Glu Tyr Asp Tyr Ile Pro Asp Ser Phe Asp Arg Glu Thr Ala Thr  
 180 185 190  
 Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val  
 195 200 205  
 Glu Leu Thr His Gln Asn Val Cys Val Arg Phe Ser His Cys Arg Asp  
 210 215 220  
 Pro Val Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Thr Val  
 225 230 235 240  
 Ile Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu  
 245 250 255  
 Thr Cys Gly Phe Arg Ile Val Leu Met Tyr Arg Phe Glu Glu Glu Leu  
 260 265 270  
 Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val  
 275 280 285  
 Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Val Asp Lys Tyr  
 290 295 300  
 Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ala  
 305 310 315 320  
 Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe Lys Leu Pro Gly Ile  
 325 330 335  
 Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Ile Ile Thr  
 340 345 350  
 Pro Glu Gly Asp Asp Lys Pro Gly Ala Cys Gly Lys Val Val Pro Phe  
 355 360 365  
 Phe Ser Ala Lys Ile Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val  
 370 375 380  
 Asn Gln Arg Gly Glu Leu Cys Val Lys Gly Pro Met Ile Met Lys Gly  
 385 390 395 400  
 Tyr Val Asn Asn Pro Glu Ala Thr Ser Ala Leu Ile Asp Lys Asp Gly  
 405 410 415  
 Trp Leu His Ser Gly Asp Ile Ala Tyr Tyr Asp Lys Asp Gly His Phe  
 420 425 430  
 Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln  
 435 440 445  
 Val Pro Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Phe Ile  
 450 455 460  
 Phe Asp Ala Gly Val Ala Gly Ile Pro Asp Pro Asp Ala Gly Glu Leu  
 465 470 475 480  
 Pro Ala Ala Val Val Val Leu Glu Glu Gly Lys Thr Met Thr Glu Gln  
 485 490 495

Glu Val Met Asp Tyr Val Ala Gly Gln Val Thr Ala Ser Lys Arg Leu  
                   500                                  505                                  510  
 Arg Gly Gly Val Lys Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly  
                   515                                  520                                  525  
 Lys Ile Asp Gly Arg Lys Ile Arg Glu Ile Leu Met Met Gly Lys Lys  
                   530                                  535                                  540  
 Ser Lys Leu  
 545

<210> 33  
 <211> 552  
 <212> PRT  
 <213> *Photuris pennsylvanica*

<400> 33  
 Met Ser Ile Glu Asn Asn Ile Leu Ile Gly Pro Pro Pro Tyr Tyr Pro  
   1                                  5                                  10                                  15  
 Leu Glu Glu Gly Thr Ala Gly Glu Gln Leu His Arg Ala Ile Ser Arg  
                   20                                  25                                  30  
 Tyr Ala Ala Val Pro Gly Thr Leu Ala Tyr Thr Asp Val His Thr Glu  
                   35                                  40                                  45  
 Leu Glu Val Thr Tyr Lys Glu Phe Leu Asp Val Thr Cys Arg Leu Ala  
                   50                                  55                                  60  
 Glu Ala Met Lys Asn Tyr Gly Leu Gly Leu Gln His Thr Ile Ser Val  
   65                                  70                                  75                                  80  
 Cys Ser Glu Asn Cys Val Gln Phe Phe Met Pro Ile Cys Ala Ala Leu  
                   85                                  90                                  95  
 Tyr Val Gly Val Ala Thr Ala Pro Thr Asn Asp Ile Tyr Asn Glu Arg  
                   100                                  105                                  110  
 Glu Leu Tyr Asn Ser Leu Ser Ile Ser Gln Pro Thr Val Val Phe Thr  
                   115                                  120                                  125  
 Ser Arg Asn Ser Leu Gln Lys Ile Leu Gly Val Gln Ser Arg Leu Pro  
                   130                                  135                                  140  
 Ile Ile Lys Lys Ile Ile Ile Leu Asp Gly Lys Lys Asp Tyr Leu Gly  
   145                                  150                                  155                                  160  
 Tyr Gln Ser Met Gln Ser Phe Met Lys Glu His Val Pro Ala Asn Phe  
                   165                                  170                                  175  
 Asn Val Ser Ala Phe Lys Pro Leu Ser Phe Asp Leu Asp Arg Val Ala  
                   180                                  185                                  190  
 Cys Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val Pro  
                   195                                  200                                  205  
 Ile Ser His Arg Asn Thr Ile Tyr Arg Phe Ser His Cys Arg Asp Pro



210	215	220
Val Phe Gly Asn Gln Ile Ile Pro Asp Thr Thr Ile Leu Cys Ala Val		
225	230	235
Pro Phe His His Ala Phe Gly Thr Phe Thr Asn Leu Gly Tyr Leu Ile		240
	245	250
Cys Gly Phe His Val Val Leu Met Tyr Arg Phe Asn Glu His Leu Phe		255
	260	265
Leu Gln Thr Leu Gln Asp Tyr Lys Cys Gln Ser Ala Leu Leu Val Pro		270
	275	280
Thr Val Leu Ala Phe Leu Ala Lys Asn Pro Leu Val Asp Lys Tyr Asp		285
	290	295
Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys		300
305	310	315
Glu Ile Ser Glu Ile Ala Ala Lys Arg Phe Lys Leu Pro Gly Ile Arg		320
	325	330
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Cys Ala Ile Val Ile Thr Ala		335
	340	345
Glu Gly Glu Phe Lys Leu Gly Ala Val Gly Lys Val Val Pro Phe Tyr		350
	355	360
Ser Leu Lys Val Leu Asp Leu Asn Thr Gly Lys Lys Leu Gly Pro Asn		365
	370	375
Glu Arg Gly Glu Ile Cys Phe Lys Gly Pro Met Ile Met Lys Gly Tyr		380
385	390	395
Ile Asn Asn Pro Glu Ala Thr Arg Glu Leu Ile Asp Glu Glu Gly Trp		400
	405	410
Ile His Ser Gly Asp Ile Gly Tyr Phe Asp Glu Asp Gly His Val Tyr		415
	420	425
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val		430
	435	440
Pro Pro Ala Glu Leu Glu Ala Leu Leu Leu Gln His Pro Phe Ile Glu		445
	450	455
Asp Ala Gly Val Ala Gly Val Pro Asp Glu Val Ala Gly Asp Leu Pro		460
465	470	475
Gly Ala Val Val Val Leu Lys Glu Gly Lys Ser Ile Thr Glu Lys Glu		480
	485	490
Ile Gln Asp Tyr Val Ala Gly Gln Val Thr Ser Ser Lys Lys Leu Arg		495
	500	505
Gly Gly Val Glu Phe Val Lys Glu Val Pro Lys Gly Phe Thr Gly Lys		510
	515	520
Ile Asp Thr Arg Lys Ile Lys Glu Ile Leu Ile Lys Ala Gln Lys Gly		525
	530	535
Lys Ser Lys Ser Lys Ala Lys Leu		540

545

550

&lt;210&gt; 34

&lt;211&gt; 546

&lt;212&gt; PRT

&lt;213&gt; Phengodes sp.

&lt;400&gt; 34

Met Ile Lys Met Glu Glu Glu His Val Met Pro Gly Ala Met Pro Arg  
 1 5 10 15  
 Asp Leu Leu Phe Glu Gly Thr Ala Gly Gln Gln Leu His Arg Ala Leu  
 20 25 30  
 Tyr Lys His Ser Tyr Phe Pro Glu Ala Ile Val Asp Ser His Thr His  
 35 40 45  
 Glu Ile Ile Ser Tyr Ala Lys Ile Leu Asp Met Ser Cys Arg Leu Ala  
 50 55 60  
 Val Ser Phe Gln Lys Tyr Gly Leu Thr Gln Asn Asn Ile Ile Gly Ile  
 65 70 75 80  
 Cys Ser Glu Asn Asn Leu Asn Phe Phe Asn Pro Val Ile Ala Ala Phe  
 85 90 95  
 Tyr Leu Gly Ile Thr Val Ala Thr Val Asn Asp Thr Tyr Thr Asp Arg  
 100 105 110  
 Glu Leu Ser Glu Thr Leu Asn Ile Thr Lys Pro Gln Met Leu Phe Cys  
 115 120 125  
 Ser Lys Gln Ser Leu Pro Ile Val Met Lys Thr Met Lys Ile Met Pro  
 130 135 140  
 Tyr Val Gln Lys Leu Leu Ile Ile Asp Ser Met Gln Asp Ile Gly Gly  
 145 150 155 160  
 Ile Glu Cys Val His Ser Phe Val Ser Arg Tyr Thr Asp Glu His Phe  
 165 170 175  
 Asp Pro Leu Lys Phe Val Pro Leu Asp Phe Asp Pro Arg Glu Gln Val  
 180 185 190  
 Ala Leu Ile Met Thr Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val  
 195 200 205  
 Met Leu Thr His Arg Asn Ile Cys Val Arg Phe Val His Ser Arg Asp  
 210 215 220  
 Pro Leu Phe Gly Thr Arg Phe Ile Pro Glu Thr Ser Ile Leu Ser Leu  
 225 230 235 240  
 Val Pro Phe His His Ala Phe Gly Met Phe Thr Thr Leu Ser Tyr Phe  
 245 250 255  
 Ile Val Gly Leu Lys Ile Val Met Met Lys Arg Phe Asp Gly Glu Leu  
 260 265 270

Phe Leu Lys Thr Ile Gln Asn Tyr Lys Ile Pro Thr Ile Val Ile Ala  
 275 280 285  
 Pro Pro Val Met Val Phe Leu Ala Lys Ser His Leu Val Asp Lys Tyr  
 290 295 300  
 Asp Leu Ser Ser Ile Lys Glu Ile Ala Thr Gly Gly Ala Pro Leu Gly  
 305 310 315 320  
 Pro Ala Leu Ala Asn Ala Val Ala Lys Arg Leu Lys Leu Gly Gly Ile  
 325 330 335  
 Ile Gln Gly Tyr Gly Leu Thr Glu Thr Cys Cys Ala Val Leu Ile Thr  
 340 345 350  
 Pro His Asn Lys Ile Lys Thr Gly Ser Thr Gly Gln Val Leu Pro Tyr  
 355 360 365  
 Val Thr Ala Lys Ile Val Asp Thr Lys Thr Gly Lys Asn Leu Gly Pro  
 370 375 380  
 Asn Gln Thr Gly Glu Leu Cys Phe Lys Ser Asp Ile Ile Met Lys Gly  
 385 390 395 400  
 Tyr Tyr Gln Asn Glu Glu Glu Thr Arg Leu Val Ile Asp Lys Asp Gly  
 405 410 415  
 Trp Leu His Ser Gly Asp Ile Gly Tyr Tyr Asp Thr Asp Gly Asn Phe  
 420 425 430  
 His Ile Val Asp Arg Leu Lys Glu Leu Ile Lys Tyr Lys Ala Tyr Gln  
 435 440 445  
 Val Ala Pro Ala Glu Leu Glu Ala Leu Leu Leu Gln His Pro Tyr Ile  
 450 455 460  
 Ala Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Glu Ala Gly Glu Leu  
 465 470 475 480  
 Pro Ala Ala Cys Val Val Leu Glu Pro Gly Lys Thr Met Thr Glu Lys  
 485 490 495  
 Glu Val Met Asp Tyr Ile Ala Glu Arg Val Thr Pro Thr Lys Arg Leu  
 500 505 510  
 Arg Gly Gly Val Leu Phe Val Asn Asn Ile Pro Lys Gly Ala Thr Gly  
 515 520 525  
 Lys Leu Val Arg Thr Glu Leu Arg Arg Leu Leu Thr Gln Arg Ala Ala  
 530 535 540  
 Lys Leu  
 545

&lt;210&gt; 35

&lt;211&gt; 543

&lt;212&gt; PRT

&lt;213&gt; Pyrophorus plagiophthalmus

&lt;400&gt; 35

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Met Met Lys Arg Glu Lys Asn Val Val Tyr Gly Pro Glu Pro Leu His
 1             5             10             15
Pro Leu Glu Asp Leu Thr Ala Gly Glu Met Leu Phe Arg Ala Leu Arg
          20             25             30
Lys His Ser His Leu Pro Gln Ala Leu Val Asp Val Tyr Gly Glu Glu
          35             40             45
Trp Ile Ser Tyr Lys Glu Phe Phe Glu Thr Thr Cys Leu Leu Ala Gln
          50             55             60
Ser Leu His Asn Cys Gly Tyr Lys Met Ser Asp Val Val Ser Ile Cys
65             70             75             80
Ala Glu Asn Asn Lys Arg Phe Phe Val Pro Ile Ile Ala Ala Trp Tyr
          85             90             95
Ile Gly Met Ile Val Ala Pro Val Asn Glu Gly Tyr Ile Pro Asp Glu
          100            105            110
Leu Cys Lys Val Met Gly Ile Ser Arg Pro Gln Leu Val Phe Cys Thr
          115            120            125
Lys Asn Ile Leu Asn Lys Val Leu Glu Val Gln Ser Arg Thr Asp Phe
          130            135            140
Ile Lys Arg Ile Ile Ile Leu Asp Ala Val Glu Asn Ile His Gly Cys
145            150            155            160
Glu Ser Leu Pro Asn Phe Ile Ser Arg Tyr Ser Asp Gly Asn Ile Ala
          165            170            175
Asn Phe Lys Pro Leu His Tyr Asp Pro Val Glu Gln Val Ala Ala Ile
          180            185            190
Leu Cys Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met Gln Thr
          195            200            205
His Arg Asn Val Cys Val Arg Leu Ile His Ala Leu Asp Pro Arg Val
          210            215            220
Gly Thr Gln Leu Ile Pro Gly Val Thr Val Leu Val Tyr Leu Pro Phe
225            230            235            240
Phe His Ala Phe Gly Phe Ser Ile Asn Leu Gly Tyr Phe Met Val Gly
          245            250            255
Leu Arg Val Ile Met Leu Arg Arg Phe Asp Gln Glu Ala Phe Leu Lys
          260            265            270
Ala Ile Gln Asp Tyr Glu Val Arg Ser Val Ile Asn Val Pro Ala Ile
          275            280            285
Ile Leu Phe Leu Ser Lys Ser Pro Leu Val Asp Lys Tyr Asp Leu Ser
          290            295            300
Ser Leu Arg Glu Leu Cys Cys Gly Ala Ala Pro Leu Ala Lys Glu Val
305            310            315            320
Ala Glu Ile Ala Val Lys Arg Leu Asn Leu Pro Gly Ile Arg Cys Gly

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	325		330		335
Phe Gly Leu Thr Glu Ser Thr Ser Ala Asn Ile His Ser Leu Arg Asp					
	340		345		350
Glu Phe Lys Ser Gly Ser Leu Gly Arg Val Thr Pro Leu Met Ala Ala					
	355		360		365
Lys Ile Ala Asp Arg Glu Thr Gly Lys Ala Leu Gly Pro Asn Gln Val					
	370		375		380
Gly Glu Leu Cys Ile Lys Gly Pro Met Val Ser Lys Gly Tyr Val Asn					
385		390		395	400
Asn Val Glu Ala Thr Lys Glu Ala Ile Asp Asp Asp Gly Trp Leu His					
	405		410		415
Ser Gly Asp Phe Gly Tyr Tyr Asp Glu Asp Glu His Phe Tyr Val Val					
	420		425		430
Asp Arg Tyr Lys Glu Leu Ile Lys Tyr Lys Gly Ser Gln Val Ala Pro					
	435		440		445
Ala Glu Leu Glu Glu Ile Leu Leu Lys Asn Pro Cys Ile Arg Asp Val					
	450		455		460
Ala Val Val Gly Ile Pro Asp Leu Glu Ala Gly Glu Leu Pro Ser Ala					
465		470		475	480
Phe Val Val Ile Gln Pro Gly Lys Glu Ile Thr Ala Lys Glu Val Tyr					
	485		490		495
Asp Tyr Leu Ala Glu Arg Val Ser His Thr Lys Tyr Leu Arg Gly Gly					
	500		505		510
Val Arg Phe Val Asp Ser Ile Pro Arg Asn Val Thr Gly Lys Ile Thr					
	515		520		525
Arg Lys Glu Leu Leu Lys Gln Leu Leu Glu Lys Ser Ser Lys Leu					
	530		535		540

&lt;210&gt; 36

&lt;211&gt; 543

&lt;212&gt; PRT

&lt;213&gt; Pyrophorus plagiophthalmus

&lt;400&gt; 36

Met Met Lys Arg Glu Lys Asn Val Ile Tyr Gly Pro Glu Pro Leu His			
1	5	10	15
Pro Leu Glu Asp Leu Thr Ala Gly Glu Met Leu Phe Arg Ala Leu Arg			
	20	25	30
Lys His Ser His Leu Pro Gln Ala Leu Val Asp Val Phe Gly Asp Glu			
	35	40	45
Ser Leu Ser Tyr Lys Glu Phe Phe Glu Ala Thr Cys Leu Leu Ala Gln			
50	55	60	

Ser Leu His Asn Cys Gly Tyr Lys Met Asn Asp Val Val Ser Ile Cys  
 65 70 75 80  
 Ala Glu Asn Asn Lys Arg Phe Phe Ile Pro Ile Ile Ala Ala Trp Tyr  
 85 90 95  
 Ile Gly Met Ile Val Ala Pro Val Asn Glu Ser Tyr Ile Pro Asp Glu  
 100 105 110  
 Leu Cys Lys Val Met Gly Ile Ser Lys Pro Gln Ile Val Phe Cys Thr  
 115 120 125  
 Lys Asn Ile Leu Asn Lys Val Leu Glu Val Gln Ser Arg Thr Asn Phe  
 130 135 140  
 Ile Lys Arg Ile Ile Ile Leu Asp Thr Val Glu Asn Ile His Gly Cys  
 145 150 155 160  
 Glu Ser Leu Pro Asn Phe Ile Ser Arg Tyr Ser Asp Gly Asn Ile Ala  
 165 170 175  
 Asn Phe Lys Pro Leu His Tyr Asp Pro Val Glu Gln Val Ala Ala Ile  
 180 185 190  
 Leu Cys Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met Gln Thr  
 195 200 205  
 His Gln Asn Ile Cys Val Arg Leu Ile His Ala Leu Asp Pro Arg Ala  
 210 215 220  
 Gly Thr Gln Leu Ile Pro Gly Val Thr Val Leu Val Tyr Leu Pro Phe  
 225 230 235 240  
 Phe His Ala Phe Gly Phe Ser Ile Asn Leu Gly Tyr Phe Met Val Gly  
 245 250 255  
 Leu Arg Val Ile Met Leu Arg Arg Phe Asp Gln Glu Ala Phe Leu Lys  
 260 265 270  
 Ala Ile Gln Asp Tyr Glu Val Arg Ser Val Ile Asn Val Pro Ala Ile  
 275 280 285  
 Ile Leu Phe Leu Ser Lys Ser Pro Leu Val Asp Lys Tyr Asp Leu Ser  
 290 295 300  
 Ser Leu Arg Glu Leu Cys Cys Gly Ala Ala Pro Leu Ala Lys Glu Val  
 305 310 315 320  
 Ala Glu Val Ala Val Lys Arg Leu Asn Leu Pro Gly Ile Arg Cys Gly  
 325 330 335  
 Phe Gly Leu Thr Glu Ser Thr Ser Ala Asn Ile His Ser Leu Gly Asp  
 340 345 350  
 Glu Phe Lys Ser Gly Ser Leu Gly Arg Val Thr Pro Leu Met Ala Ala  
 355 360 365  
 Lys Ile Ala Asp Arg Glu Thr Gly Lys Ala Leu Gly Pro Asn Gln Val  
 370 375 380  
 Gly Glu Leu Cys Val Lys Gly Pro Met Val Ser Lys Gly Tyr Val Asn  
 385 390 395 400

Asn Val Glu Ala Thr Lys Glu Ala Ile Asp Asp Asp Gly Trp Leu His  
 405 410 415  
 Ser Gly Asp Phe Gly Tyr Tyr Asp Glu Asp Glu His Phe Tyr Val Val  
 420 425 430  
 Asp Arg Tyr Lys Glu Leu Ile Lys Tyr Lys Gly Ser Gln Val Ala Pro  
 435 440 445  
 Ala Glu Leu Glu Glu Ile Leu Leu Lys Asn Pro Cys Ile Arg Asp Val  
 450 455 460  
 Ala Val Val Gly Ile Pro Asp Leu Glu Ala Gly Glu Leu Pro Ser Ala  
 465 470 475 480  
 Phe Val Val Lys Gln Pro Gly Lys Glu Ile Thr Ala Lys Glu Val Tyr  
 485 490 495  
 Asp Tyr Leu Ala Glu Arg Val Ser His Thr Lys Tyr Leu Arg Gly Gly  
 500 505 510  
 Val Arg Phe Val Asp Ser Ile Pro Arg Asn Val Thr Gly Lys Ile Thr  
 515 520 525  
 Arg Lys Glu Leu Leu Lys Gln Leu Leu Glu Lys Ser Ser Lys Leu  
 530 535 540

&lt;210&gt; 37

&lt;211&gt; 545

&lt;212&gt; PRT

<213> *Photuris pennsylvanica*

&lt;400&gt; 37

Met Glu Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu  
 1 5 10 15  
 Ala Asp Gly Thr Ala Gly Glu Gln Met Phe Tyr Ala Leu Ser Arg Tyr  
 20 25 30  
 Ala Asp Ile Ser Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu  
 35 40 45  
 Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu  
 50 55 60  
 Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys  
 65 70 75 80  
 Ser Glu Asn Gly Leu Gln Phe Phe Leu Pro Leu Ile Ala Ser Leu Tyr  
 85 90 95  
 Leu Gly Ile Ile Ala Ala Pro Val Ser Asp Lys Tyr Ile Glu Arg Glu  
 100 105 110  
 Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Ile Phe Cys Ser  
 115 120 125  
 Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Tyr

130	135	140
Val Glu Thr Ile Ile Ile Leu Asp Leu Asn Glu Asp Leu Gly Gly Tyr		
145	150	155
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ile Asn Leu Asp		160
	165	170
Val Lys Lys Phe Lys Pro Asn Ser Phe Asn Arg Asp Asp Gln Val Ala		175
	180	185
Leu Val Met Phe Ser Ser Gly Thr Thr Gly Val Ser Lys Gly Val Met		190
	195	200
Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser His Cys Lys Asp Pro		205
	210	215
Thr Phe Gly Asn Ala Ile Asn Pro Thr Thr Ala Ile Leu Thr Val Ile		220
225	230	235
Pro Phe His His Gly Phe Gly Met Thr Thr Thr Leu Gly Tyr Phe Thr		240
	245	250
Cys Gly Phe Arg Val Ala Leu Met His Thr Phe Glu Glu Lys Leu Phe		255
	260	265
Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro		270
	275	280
Thr Leu Met Ala Phe Phe Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp		285
	290	295
Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys		300
305	310	315
Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg		320
	325	330
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro		335
	340	345
Asp Thr Asp Val Arg Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His		350
	355	360
Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn		365
	370	375
Glu Thr Gly Glu Leu Tyr Phe Lys Gly Asp Met Ile Met Lys Ser Tyr		380
385	390	395
Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asn Lys Asp Gly Trp		400
	405	410
Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr		415
	420	425
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val		430
	435	440
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val		445
	450	455
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro		460



465                      470                      475                      480  
 Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile  
                                  485                      490                      495  
 Val Gln Asn Phe Val Ser Ser Gln Val Ser Thr Ala Lys Trp Leu Arg  
                                  500                      505                      510  
 Gly Gly Val Lys Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys  
                                  515                      520                      525  
 Ile Asp Arg Lys Val Leu Arg Gln Met Phe Glu Lys His Lys Ser Lys  
                                  530                      535                      540  
 Leu  
 545

<210> 38  
 <211> 38  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> /note = "a primer"

<400> 38  
 gtactgagac gacgccagcc caagcttagg cctgagtg

38

<210> 39  
 <211> 38  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> /note = "a primer"

<400> 39  
 ggcatgagcg tgaactgact gaactagcgg ccgccgag

38

<210> 40  
 <211> 18  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> /note = "a primer"

<400> 40  
 gtactgagac gacgccag 18

<210> 41  
 <211> 19  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> /note = "a primer"

<400> 41  
 ggcatgagcg tgaactgac 19

<210> 42  
 <211> 1639  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> /note = "mutant luciferase"

<400> 42  
 agatccaatg gcagataaga atatTTtata tgggcccga ccatTTtatc ccttggaaga 60  
 tgggacggct ggagaacaga tgTTtgacgc attatctcgt tatgcagata ttccgggctg 120  
 catagcattg acaaatgctc atacaaaaga aaatgTTtta tatgaagagt ttctgaaact 180  
 gtcgtgtcgt ttagcggaaa gTTTTaaaa gtatggatta aaacaaaacg acacaatagc 240  
 ggtgtgtagc gaaaatagtc tgcaatTTTT cTTTcctgta attgcatcat tgtatcTTgg 300  
 aataattgtg gcacctgtta acgataaata cattgaacgt gaattaatac acagtcttgg 360  
 tattgtaaaa ccacgcatag tTTTTtgctc caagaatact tttcaaaaag tactgaatgt 420  
 aaaatctaaa ttaaaatcta ttgaaactat tattatatta gacttaaatg atgacttagg 480  
 aggttatcaa tgctcaaca actTTatttc tcaaaattcc gatagtaatc tggacgtaaa 540  
 aaaatttaaa ccatattctt ttaatcgaga cgatcagggtt gogttgatta tgtTTTcttc 600  
 tgggtacaact ggtctgcga agggagtcac gctaactcac aagaatattg ttgcacgatt 660  
 ttctattgca aaagatccta cTTTtggtta cgcaattaat ccacgtcag caattTTaac 720  
 ggtaatacct ttccaccatg gTTTtggtat gatgaccaca ttaggatact ttacttTgtg 780  
 attccgagtt gttctaattgc acacgTTtga agaaaaacta tttctacaat cattacaaga 840  
 ttataaagtg gaaagtactt tacttTgtacc aacattaatg gcattTcttg caaaaagtgc 900  
 attagttgaa aagtacgatt tatcgactt aaaagaaatt gcatctggTg ggcacctTT 960  
 atcaaaagaa attggggaga tggTgaaaa acggtTTtaa ttaaactTTg tcaggcaagg 1020  
 gtatggatta acagaaacca cTtcggctgt tTTaattaca ccgaaaggTg acgccaaacc 1080  
 gggatcaact ggtaaaatag taccattTca cgctgtTaaa gTtTgcgatc ctacaacagg 1140

aaaaattttg	gggccaaatg	aacctggaga	attgtatttt	aaaggcccga	tgataatgaa	1200
gggttattat	aataatgaag	aagctactaa	agcaattatt	gataatgacg	gatggttgcg	1260
ctctggtgat	attgcttatt	atgacaatga	tggccatttt	tatattgtgg	acaggctgaa	1320
gtcactgatt	aaatataaag	gttatcaggt	tgcacctgct	gaaattgagg	gaatactctt	1380
acaacatccg	tatattgttg	atgccggcgt	tactggtata	ccggatgaag	ccgcgggcga	1440
gcttcagct	gcagggtgtg	tagtacagac	tggaaaatat	ctaaacgaac	aaatcgtaca	1500
agattatggt	gccagtcaag	tttcaacagc	caaattggcta	cgtgggtggg	tgatattttt	1560
ggatgaaatt	cccaaaggat	caactggaaa	aattgacaga	aaagtgttaa	gacaaatggt	1620
agaaaaacac	accaatggg					1639

&lt;210&gt; 43

&lt;211&gt; 1639

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;400&gt; 43

ggatccaatg	gcagataaga	atattttata	tgggcccga	ccattttatc	ccttgggaaga	60
tgggacggct	ggagaacaga	tgtttgacgc	attatctcgt	tatgcagcta	ttccgggctg	120
catagcattg	acaaatgctc	atacaaaaaga	aaatgtttta	tatgaagagt	ttctgaaact	180
gtcgtgtcgt	ttagcggaaa	gttttaaaaa	gtatggatta	aaacaaaacg	acacaatagc	240
ggtgtgtagc	gaaaaatagtc	tgcaattttt	ccttcctgta	attgcatcat	tgtatcttgg	300
aataattgtg	gcacctgtta	acgataaata	cattgaacgt	gaattaatac	acagtcttgg	360
tattgtaaaa	ccacgcatag	ttttttgctc	caagaatact	tttcaaaaag	tactgaatgt	420
aaaatctaaa	ttaaaatcta	ttgaaactat	tattatatta	gacttaaatg	aagacttagg	480
aggttatcaa	tgccctcaaca	actttatttc	tcaaaattcc	gatagtaatc	tggacgtaaa	540
aaaattttaa	ccctattctt	ttaatcgaga	cgatcagggt	gcgtcgatta	tgttttcttc	600
tggatcaact	ggtctgccga	agggagtcac	gctaactcac	aagaatattg	ttgcacgatt	660
ttctattgca	aaagatccta	cttttggtta	cgcaattaat	cccacgtcag	caattttaac	720
ggtaatacct	ttccaccatg	gttttggtat	gatgaccaca	ttaggatact	ttacttgtgg	780
attccgagtt	gttctaatac	acacgtttga	agaaaaacta	tttctacaat	cattacaaga	840
ttataaagtg	gaaagtactt	tacttgtacc	aacattaatg	gcatttcttg	caaaaagtgc	900
attagttgaa	aagtacgatt	tatcgcactt	aaaagaaatt	gcacgtggtg	gcgcaccttt	960
atcaaaagaa	attggggaga	tggtgaaaaa	acggttttaa	ttaaactttg	tcaggcaagg	1020
gtatggatta	acagaaacca	cttcggctgt	tttaattaca	ccgaaagggtg	acgccaaacc	1080
gggatcaact	ggtaaaatag	taccattaca	cgctgtttaa	gttgtcgatc	ctacaacagg	1140
aaaaattttg	gggccaaatg	aacctggaga	attgtatttt	aaaggcccga	tgataatgaa	1200
gggttattat	aataatgaag	aagctactaa	agcaattatt	gataatgacg	gatggttgcg	1260
ctctggtgat	attgcttatt	atgacaatga	tggccatttt	tatattgtgg	acaggctgaa	1320
gtcactgatt	aaatataaag	gttatcaggt	tgcacctgct	gaaattgagg	gaatactctt	1380

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acaacatccg tatattgttg atgccggcgt tactggtata ccggatgaag ccgcgggcga      1440
gcttccagct gcaggtgttg tagtacagac tggaaaatat ctaaacgaac aaatcgtaca      1500
agattatggt gccagtcaag tttcaacagc caaatggcta cgtggtgggg tgaaattttt      1560
ggatgaaatt cccaaaggat caactggaaa aattgacaga aaagtgttaa gacaaatggt      1620
agaaaaacac accaatggg                                     1639

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<210> 44

<211> 544

<212> PRT

<213> Artificial Sequence

<220>

<223> /note = "mutant luciferase"

<400> 44

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Met Ala Asp Lys Asn Ile Leu Tyr Gly Pro Glu Pro Phe Tyr Pro Leu
 1             5             10             15
Glu Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr
          20             25             30
Ala Asp Ile Pro Gly Cys Ile Ala Leu Thr Asn Ala His Thr Lys Glu
      35             40             45
Asn Val Leu Tyr Glu Glu Phe Leu Lys Leu Ser Cys Arg Leu Ala Glu
      50             55             60
Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
65             70             75             80
Ser Glu Asn Ser Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr
          85             90             95
Leu Gly Ile Ile Val Ala Pro Val Asn Asp Lys Tyr Ile Glu Arg Glu
          100             105             110
Leu Ile His Ser Leu Gly Ile Val Lys Pro Arg Ile Val Phe Cys Ser
          115             120             125
Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Ser
          130             135             140
Ile Glu Thr Ile Ile Ile Leu Asp Leu Asn Asp Asp Leu Gly Gly Tyr
145             150             155             160
Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ser Asn Leu Asp
          165             170             175
Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
          180             185             190
Leu Ile Met Phe Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met
          195             200             205
Leu Thr His Lys Asn Ile Val Ala Arg Phe Ser Ile Ala Lys Asp Pro

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210	215	220
Thr Phe Gly Asn Ala Ile Asn Pro Thr Ser Ala Ile Leu Thr Val Ile		
225	230	235
Pro Phe His His Gly Phe Gly Met Met Thr Thr Leu Gly Tyr Phe Thr		240
	245	250
Cys Gly Phe Arg Val Val Leu Met His Thr Phe Glu Glu Lys Leu Phe		255
	260	265
Leu Gln Ser Leu Gln Asp Tyr Lys Val Glu Ser Thr Leu Leu Val Pro		270
	275	280
Thr Leu Met Ala Phe Leu Ala Lys Ser Ala Leu Val Glu Lys Tyr Asp		285
	290	295
Leu Ser His Leu Lys Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys		300
305	310	315
Glu Ile Gly Glu Met Val Lys Lys Arg Phe Lys Leu Asn Phe Val Arg		320
	325	330
Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Val Leu Ile Thr Pro		335
	340	345
Lys Gly Asp Ala Lys Pro Gly Ser Thr Gly Lys Ile Val Pro Phe His		350
	355	360
Ala Val Lys Val Val Asp Pro Thr Thr Gly Lys Ile Leu Gly Pro Asn		365
	370	375
Glu Pro Gly Glu Leu Tyr Phe Lys Gly Pro Met Ile Met Lys Gly Tyr		380
385	390	395
Tyr Asn Asn Glu Glu Ala Thr Lys Ala Ile Ile Asp Asn Asp Gly Trp		400
	405	410
Leu Arg Ser Gly Asp Ile Ala Tyr Tyr Asp Asn Asp Gly His Phe Tyr		415
	420	425
Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val		430
	435	440
Ala Pro Ala Glu Ile Glu Gly Ile Leu Leu Gln His Pro Tyr Ile Val		445
	450	455
Asp Ala Gly Val Thr Gly Ile Pro Asp Glu Ala Ala Gly Glu Leu Pro		460
465	470	475
Ala Ala Gly Val Val Val Gln Thr Gly Lys Tyr Leu Asn Glu Gln Ile		480
	485	490
Val Gln Asp Tyr Val Ala Ser Gln Val Ser Thr Ala Lys Trp Leu Arg		495
	500	505
Gly Gly Val Ile Phe Leu Asp Glu Ile Pro Lys Gly Ser Thr Gly Lys		510
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&lt;210&gt; 45

&lt;211&gt; 544

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; /note = "mutant luciferase"

&lt;400&gt; 45

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Glu Asp Gly Thr Ala Gly Glu Gln Met Phe Asp Ala Leu Ser Arg Tyr
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Ser Phe Lys Lys Tyr Gly Leu Lys Gln Asn Asp Thr Ile Ala Val Cys
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Ser Glu Asn Ser Leu Gln Phe Phe Leu Pro Val Ile Ala Ser Leu Tyr
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Leu Gly Ile Ile Val Ala Pro Val Asn Asp Lys Tyr Ile Glu Arg Glu
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Lys Asn Thr Phe Gln Lys Val Leu Asn Val Lys Ser Lys Leu Lys Ser
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Gln Cys Leu Asn Asn Phe Ile Ser Gln Asn Ser Asp Ser Asn Leu Asp
          165             170             175
Val Lys Lys Phe Lys Pro Tyr Ser Phe Asn Arg Asp Asp Gln Val Ala
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          195             200             205
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&lt;210&gt; 46

&lt;211&gt; 1633

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

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			85					90					95		
Ile	Gly	Met	Ile	Val	Ala	Pro	Val	Asn	Glu	Ser	Tyr	Ile	Pro	Asp	Glu
	100							105					110		
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Phe Val Val Lys Gln Pro Gly Lys Glu Ile Thr Ala Lys Glu Val Tyr		
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&lt;211&gt; 23

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

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&lt;211&gt; 22

&lt;212&gt; DNA

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25

## INTERNATIONAL SEARCH REPORT

Internat Application No  
PCT/US 99/30925

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/53 C12N15/10 C12N9/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CAB Data, STRAND, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 99 14336 A (HALL MARY P ; PROMEGA CORP (US); WOOD KEITH V (US)) 25 March 1999 (1999-03-25)</p> <p>Fig.46,47 page 34, line 1 -page 42, line 6; table 6 page 47, line 3 -page 61, line 2 --- -/--</p>	<p>1-6,8, 12,14, 16,17, 19,21, 23,24, 28-37, 47-58, 65,66, 68,73,75</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

7 August 2000

Date of mailing of the international search report

14.08.00

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Hornig, H

## INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/US 99/30925

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 524 448 A (KIKKOMAN CORP) 27 January 1993 (1993-01-27)  the whole document ---	1-5,8, 12,14, 16,17, 19,21, 23,24, 28-37, 58,65, 66,73
X	WO 95 25798 A (SECR DEFENCE BRIT ;LOWE CHRISTOPHER ROBIN (GB); WHITE PETER JOHN ( ) 28 September 1995 (1995-09-28)  the whole document ---	1-5,8, 12,14, 16,17, 19,21, 23,24, 28-37, 58,65, 66,73
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## INTERNATIONAL SEARCH REPORT

 Intern:      if Application No  
 PCT/US 99/30925

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>K.V. WOOD ET AL.: "Complementary DNA coding click beetle luciferases can elicit bioluminescence of different colors"            SCIENCE,            vol. 244, 12 May 1989 (1989-05-12), pages 700-702, XP002137278            AAAS, WASHINGTON, DC, US            cited in the application            the whole document</p> <p>---</p>	
A	<p>K.V. WOOD ET AL.: "Introduction to beetle luciferases and their applications"            J. BIOLUMINESCENCE AND CHEMILUMINESCENCE,            vol. 4, no. 1, July 1989 (1989-07), pages 289-301, XP000906968            JOHN WILEY &amp; SONS, LTD, NEW YORK, US            cited in the application            the whole document</p> <p>---</p>	
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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	<p>CADWELL R AND JOYCE G ET AL: "Randomization of genes by PCR mutagenesis"</p> <p>PCR METHODS AND APPLICATIONS,US,COLD SPRING HARBOR, NY, vol. 2, 1992, pages 28-33, XP002087462</p> <p>ISSN: 1054-9803</p> <p>the whole document</p> <p>---</p>	47-57,75
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# INTERNATIONAL SEARCH REPORT

Int ional application No.  
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## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 76 (11-partially)  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
In claim 11, SEQ ID Nos. 12 and 13 do not encode any amino acid sequence of claim 9 and 10.  
Claim 76 refers to itself.
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: (1-10,12-46,65-68,73)-complete, (11,58)-partially

A second luciferase that has enhanced resistance to an inhibitor of the luciferase relative to a first reference beetle luciferase; said second luciferase which comprises a plurality of amino acid substitutions relative to the reference beetle luciferase; said second luciferase wherein the reference luciferase is LucpPe-2 (luciferase from *Photuris pennsylvanica*); luciferases: luc 133-1B2 and luc146-1H2 which comprise SEQ ID Nos. 44 and 45; an isolated and purified nucleic acid molecule comprising a nucleic acid sequence selected from SEQ ID Nos. 42 and 43; a vector comprising said nucleic acid molecule; a host cell comprising said vector; a fusion protein comprising said luciferase; the use of the second luciferase for detecting ATP, for labeling a molecule, as a genetic reporter, for immobilization onto a solid surface, to produce a hybrid molecule, for high temperature reactions, or for creating luminescent solutions; a method for using said vector encoding said luciferase; a kit comprising: a container comprising said second luciferase; method to prepare an luciferase that is resistant to an inhibitor, comprising: a) selecting one or more isolated polynucleotide sequences encoding luciferase which is resistant to an inhibitor from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding luciferase subjected to conditions that yield nucleotide mutations, wherein the luciferase encoded by the one or more selected isolated polynucleotide sequences has increased resistance to an inhibitor relative to the luciferase encoded by the first isolated polynucleotide sequence; b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences; and c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding luciferase that is resistant to an inhibitor and comprises a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence; said method wherein the first polynucleotide sequence encodes Ppe2 and/or Pp1;

1.1. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc49-7C6, respectively SEQ ID Nos. 1 and 14, where the reference beetle luciferase is lucPpe-2;

1.2. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc78-0B10, respectively SEQ ID Nos. 6 and 19, where the reference beetle luciferase is lucPpe-2;

- 1.3. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc90-1B5 respectively SEQ ID Nos. 11 and 24, where the reference beetle luciferase is lucPpe-2;

- 1.4. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc81-6G01 respectively SEQ ID Nos. 13 and 26, where the reference beetle luciferase is lucPpl;

- 1.5. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc81-0B11, respectively SEQ ID Nos. 46 and 47, where the reference beetle luciferase is lucPpl;

- 1.6. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to lucPpe-2[T249M], respectively SEQ ID No. 12, where the reference beetle luciferase is lucPpe-2;

2. Claims: 47-57,75

A method to prepare an enzyme which is not a beetle luciferase and which has enhanced enzymological properties, comprising: a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is not a luciferase and which has at least one enhanced enzymological property from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme which is not a luciferase, wherein the first isolated polynucleotide sequence is subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has at least one enhanced enzymological property relative to the enzyme encoded by the first isolated polynucleotide sequence; b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences, wherein the selected isolated polynucleotide

sequence is subjected to oligonucleotide mediated mutagenesis with a plurality of oligonucleotides each comprising at least one codon that encodes a consensus amino acid which is not present in the first polynucleotide sequence; and c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme which is not a luciferase having at least one enhanced enzymological property and comprising a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence; said method wherein the enzyme is DNA polymerase or RNA polymerase, chloramphenicol-acetyltransferase, beta-glucuronidase or beta-galactosidase; an enzyme which is encoded by the polynucleotide sequence of said method;

3. Claims: 58-partially, (59-64,69-72,74)-complete

A method to prepare an enzyme that is resistant to an inhibitor, comprising: a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is resistant to an inhibitor from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has increased resistance to an inhibitor relative to the enzyme encoded by the first isolated polynucleotide sequence; b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences; and c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme that is resistant to an inhibitor and comprises a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence; said method wherein the further polynucleotide sequence encodes an enzyme that has increased thermostability relative to the first polynucleotide sequence, said method wherein said enzyme is DNA or RNA polymerase; a polynucleotide sequence obtained by said m

Please note that all inventions mentioned under item 1, although not necessarily linked by a common inventive concept, could be searched without effort justifying an additional fee.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

Continuation of Box I.2

Claims Nos.: 76 (11-partially)

In claim 11, SEQ ID Nos. 12 and 13 do not encode any amino acid sequence of claim 9 and 10.

Claim 76 refers to itself.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat Application No

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